

Laboratory-based aerobic denitrification  
using  
methanotrophic biomass as substrate

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## Abstract

Nitrate levels in our water resources have doubled since 20<sup>th</sup> century. This is largely due to anthropogenic activities such as overuse of nitrogen fertilizers, combustion of fossil fuels, and improper disposal of human and animal waste. Drinking water with high nitrate concentration can be highly toxic and is known to be associated with methemoglobinemia and cancer. Combined with the nature of nitrate ion ( $\text{NO}_3^-$ ) to be readily soluble in water, it is important to develop infrastructure to control the release of nitrates in the ecosystem.

Various conventional methods currently used to remove nitrate from water include ion exchange, distillation and reverse osmosis. However, these methods show poor selectivity towards nitrate removal and are expensive to operate. One of the promising alternative strategies is to implement a bioremediation process that uses denitrifying bacteria. Denitrifying bacteria are able to convert nitrates in soil into atmospheric nitrogen. Harnessing the natural ability of these microorganisms would be a cost-sensitive and a low-maintenance approach to control the release of nitrates in water bodies.

Researchers at ESR and Chemical and Process Engineering (CAPE - University of Canterbury) are currently focused towards developing innovative approaches to reduce nitrate in the aquifer by promoting denitrifying bacterial growth in situ. This thesis focuses on this noble cause by exploring the denitrification capabilities of several denitrifying bacteria across different media compositions. The denitrifying bacteria studied in these experiments in the thesis were *Bacillus subtilis*, *Paracoccus denitrificans*, *Acidovorax spp.*, *Paenibacillus macerans* and *Pseudomonas stutzeri*. Among different media tested, we also test the denitrification capacity of these bacteria when it uses methanotroph biomass as a carbon and energy source. We demonstrate that aerobic denitrification can be observed with all the media tested in this study - Lysogeny broth (LB), denitrification media, and also with modified media with yeast extract and methanotrophic biomass as carbon and energy sources. The highest amount of nitrate reduction was observed with LB medium and sub-optimal nitrate reduction was observed with yeast extract and methanotrophic biomass. Given that denitrification is a low energy yielding process, it makes sense for the denitrification to be suboptimal with complex media. This knowledge can be further explored to identify an economically viable optimal media that can be used for biological denitrification of waste-water treatment.

## Objectives

The primary objective of this research was to study the denitrification products formed during the growth cycle of denitrifying bacteria and make initial inferences about relations between biomass and reduction of nitrate. The second objective was to grow the denitrifiers with methanotrophic biomass, serving as a growth medium and to test for reduction in concentration of nitrate and concentration of other products formed. The third objective was to repeat the same experiment by using a live methanotrophic culture. Growing methanotrophs and denitrifiers together and analyzing the products formed during denitrification, if the process took place.

## Specific Aims

1. To explore the various denitrification products produced by strains in study (denitrifiers) - *Bacillus subtilis*, *Paracoccus denitrificans*, *Acidovorax spp.*, *Paenibacillus macerans* and *Pseudomonas stutzeri* during the process of denitrification.
2. To explore the relationship between reduction of nitrate and live methanotrophs/methanotrophic biomass when the denitrifiers are cultured in medium with biomass as the carbon and energy source
3. To test for changes in the concentration of nitrate, nitrite and ammonia when methanotrophs and its biomass is cultured with denitrifiers

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# CHAPTER ONE

## Literature Review

## PART ONE: DENITRIFIERS

### 1.1 Nitrate prevalence in the environment

Nitrate is a salt that occurs naturally when oxygen and nitrogen combine and can exist in the atmosphere or dissolved in water [1], [2]. Nitrates ( $\text{NO}_3^-$ ) are prevalent in the environment in both - organic and inorganic forms. In nature, nitrates are generally formed when bacteria act upon compounds that contain nitrogen [3].

The presence of nitrate is known to affect the quality of water in many ways [3]. The applied nitrogen drains from agricultural fields to contaminate fresh and ground water [4]. In Europe the nitrate levels differed based on the depth of the aquifer<sup>1</sup>. Shallow aquifers with the highest levels of nitrate 26.5 mg/L followed by deeper aquifers 17.7 mg/L [5]. In New Zealand, nitrate leaching is shown highest in Waikato (north island), Manawatū-Wanganui and Canterbury regions (south island) and has remained fairly constant with the exception of Canterbury [2], where it has doubled over time [6] (Fig 1.1).

Excess nutrients in water-bodies like lakes and rivers can favor the biological growth of certain organisms, disturbing the balance of the ecosystem. Some environmental studies have evaluated the levels of nitrates in water bodies and have observed higher than normal levels, making nitrates a critical environmental concern (Kovac et al., 2018).

The global deposition of nitrogen in the land has doubled since 1920 due to the practice of use of nitrogen fertilizers (inorganic, manure) and rapid use of fossil fuels [4]. The increase in nitrate contamination is widely associated with a multitude of sources like nitrogen fertilizers, human excrement, livestock and other organic waste [7], [8]. The ever-increasing concentration of nitrate has become a global issue. The maximum contaminant level (MCL) for nitrates in the United States and New Zealand for drinking water for public is 10 mg/L, which is nearly equivalent to the regulatory limit set by WHO which is 11.3 mg/L [3].

This increase in nitrate levels gives rise to conditions that disturb the ecological balance and make it challenging for the survival of fishes, insects and plants. Even though nitrites<sup>2</sup> are short-

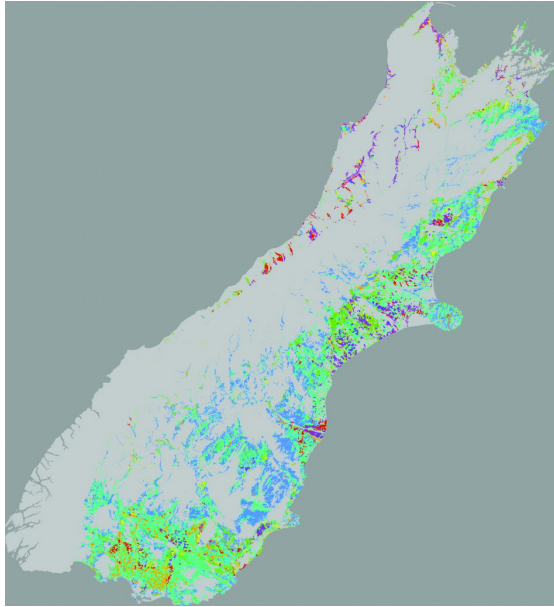
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<sup>1</sup> Aquifers are underground layers of porous rock or sand through which groundwater flows, and through this process it accumulates several minerals including nitrates (Pawson, 2008)

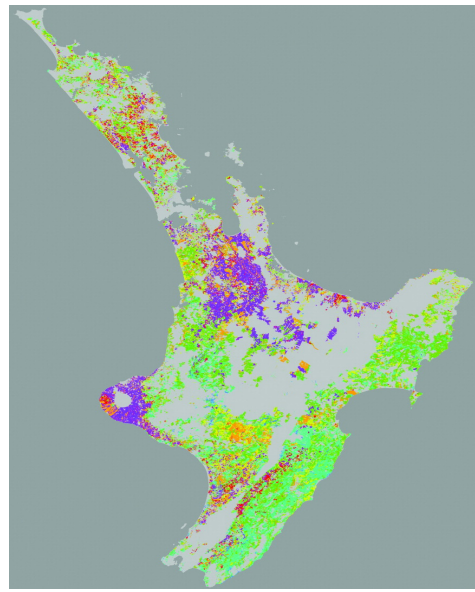
<sup>2</sup> Nitrite - oxidized form of nitrate and the reduced form of nitric oxide, bioactive molecule

lived as they quickly convert to nitrates, they manage to cause serious illness like the “brown blood disease” in fishes [3]. It also reacts with hemoglobin in blood to create met-hemoglobin which renders the ability of blood to transport oxygen inefficient. This condition is widely referred to as the “blue baby” disease or methemoglobin. Although the nitrate levels that affect infants do not cause harm to older children or adults [3]. The presence of nitrates does not have a direct effect on aquatic life, however it can give rise to conditions that make survival of fishes, insects and plants challenging. Cyanobacteria are known to grow at an exponential rate in presence of high concentrations of nitrogen in water. According to studies conducted by [9] cyanobacterial genera (*Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya*, *Microcystis*, *Nodularia*, and *Nostoc*) hamper the quality of water by forming dense blooms, scums and mats [6]. These cyanobacteria are known to produce toxins (cytotoxins and biotoxins) in both freshwater and brackish water reservoirs and are responsible for poisoning of animals (domestic and wild) and humans [10]. As time passes the cyanobacteria die and decompose, depleting the oxygen and leading to death of other organisms. An efficient way to manage groundwater contamination is to understand the origin and transport of nitrates through aquifers.

(A)



(B)

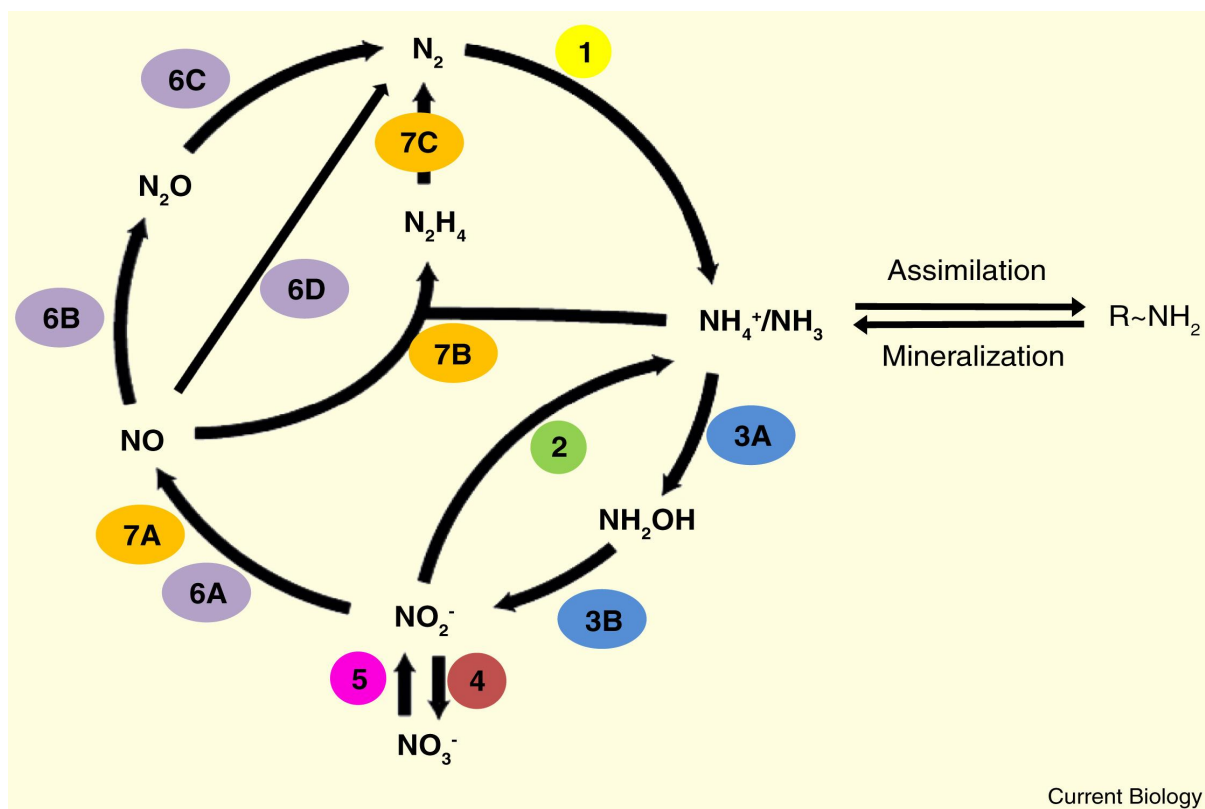


**Fig 1.1: A national map of New Zealand showing rates of nitrate leaching into soil**

(A) The south island and (B) the north island of NZ [6]. The colors represent rates of leaching ( $\text{kg nitrate-N ha}^{-1} \text{ yr}^{-1}$ ) grey (0–2), blue (2–5), teal (5–10), olive green (10–15), yellow (15–20), orange (20–30), red (30–40) and purple ( $>40$ ) .

## 1.2 Nitrogen cycle

There are three participating processes that make up the classical nitrogen cycle- nitrogen fixation, nitrification and denitrification. The microbes involved in a process have been identified and named according to the process they are associated with, for example “nitrogen fixers” for microbes that fix nitrogen, “nitrifiers” that are involved in nitrification and “denitrifiers” that are sole players in the process of denitrification [11]. These microorganisms form a complex network linking all the nitrogen transforming reactions together that do not take place in an orderly fashion [12]. There are several ways in which one process can be accomplished as shown in the figure below.



**Fig 1.2: A graphical representation of the nitrogen cycle**

Ammonification process 1 depicts ammonification, process 2 portrays nitrogen fixation, process 3 (3A- ammonia to hydroxylamine, 3B- hydroxylamine to nitrous oxide) highlights nitrification (oxidation of ammonia to nitrite), and process 4 is composed of oxidation of nitrite to nitrate. Process 5 (nitrate to nitrite) and 6 (6A- nitrite to nitric oxide, 6B- nitric oxide to nitrous oxide, 6C- nitrous oxide to nitrogen gas, 6D- nitric oxide to nitrogen gas) shows denitrification. Process 7 is an alternative method via which denitrification can be achieved (7A- nitric oxide to hydrazine, 7B- nitric oxide to ammonia, 7C- hydrazine to nitrogen gas) [13].

### **1.3 Conventional physical, chemical and bioremediation processes**

The conventional methods used for making water drinkable are reverse osmosis, nano-filtration, electro-dialysis, and distillation. However, these methods show poor selectivity towards removal of nitrates, thus demanding chemical processes for purifying water [14]. This can easily get expensive, and moreover using chemical processes such as ion exchange that purify water of nitrates, chlorides, and sulphates produce rich brines of these chemicals that are burdensome to dispose of [15].

Alternative purification processes involve bioremediation where plants and microorganisms are used for detoxification of contaminants from this environment [16]. As this research field is not totally explored, my thesis focuses on identifying and characterizing microorganisms that can aid in the process of denitrification. Denitrifying bacteria are free-living bacteria that can be found in the environment and are known to convert nitrate to gaseous nitrogen and nitrous oxide [17].

### **1.4 A road map to understanding denitrification**

Denitrification also known as nitrate respiration is the dissimilatory reduction of nitrate to form molecular (gaseous) nitrogen or oxides of nitrogen like nitric oxide [18,19]. The two important steps in this process is the conversion of nitrate to nitrite (first step) and nitrite to nitrogen gas or ammonium through intermediates (second step) [20].

The requirement of energy and cell synthesis in these biochemically and taxonomically diverse microorganisms are met by utilisation of a reduced organic substrate. This process is carried out by a number of bacteria that use oxidised nitrogen compounds as terminal electron acceptors in the absence of oxygen [21]. Most of these nitrate reducing bacteria are heterotrophs, while some survive on single carbon compounds and others on hydrogen, carbon dioxide and sulfur compounds [22].

The oxidation of these organic matter involves molecular oxygen, which is involved in the oxidation of organic matter and hence is the most common and crucial electron acceptor on



earth [23]. In anaerobic conditions, bacteria are able to use several other electron acceptors, one of the most common one being nitrate as its oxidative potential approximates that of oxygen [23].

## **1.5 Comparison of aerobic, intra-aerobic respiration and denitrification**

The same core machinery is responsible for aerobic respiration and denitrification and serves as a backbone for the different modules involving NADH dehydrogenase (complex I), the quinone pool, the bc<sub>1</sub> complex (complex III) and cytochrome c. These pathways - denitrification and respiration add their own specific modules to this framework [23]. The addition of yet another pathway due to dismutation of nitric oxide ( $2\text{NO} \rightarrow \text{N}_2 + \text{O}_2$ ) leads to hybrid denitrification also known as intra-aerobic denitrification. No electrons are required for the dismutation of NO, such a chain starts with reduction of nitrate or nitrite to nitric oxide, where nitric oxide goes through simultaneous oxidation and reduction into oxygen and the final step of the reaction proceeds aerobically [24].

## 1.6 The world of denitrifying bacteria

The growth of denitrifying bacteria and its nature of nitrate removal is influenced by a number of environmental and operating factors like carbon source, pH and temperature [25]. These denitrifying bacteria are generally known to carry out a stepwise reduction of nitrates and there are a number of known pathways based on the products formed. Denitrification, nitrate assimilation or ammonification (converting organic N to ammonia) are a few of the well-studied pathways [26], [27].

### 1.6.1 Chemical reactions

Two interrelated biochemical reactions are accountable for anaerobic denitrification, metabolism of a carbon source via the tricarboxylic acid (TCA) cycle with the production of NADH and ATP, and use of the NADH produced to biologically reduce nitrate [28].

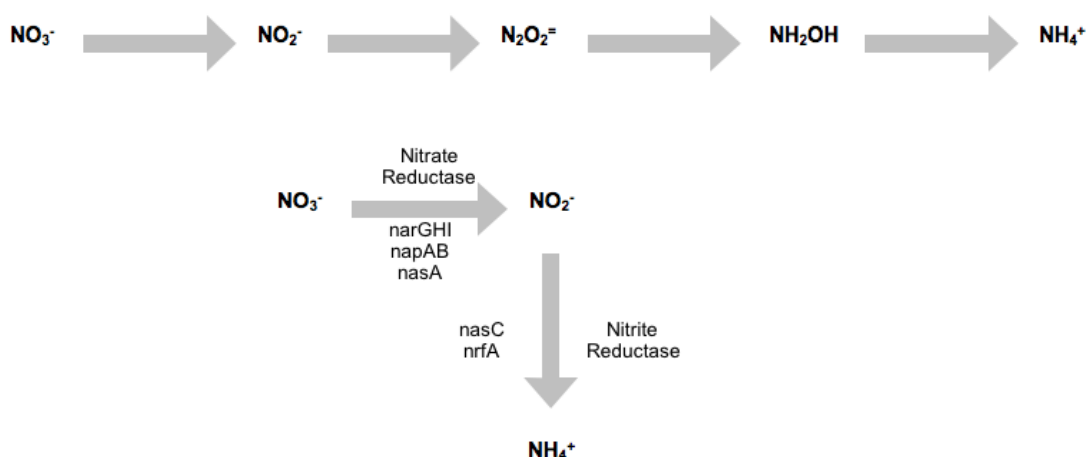
#### 1.6.1.1 Denitrification



**Fig 1.3: Denitrification pathway**

Nitrate is broken down to nitrite, the key enzyme in this conversion is nitrate reductase and the genes involved are narGHI, napAB, nasA. This step is followed by the breakdown of nitrite to nitric oxide and breakdown of nitric oxide to nitrous oxide. Nitrite reductase plays a key role in both these transformations and the genes involved are nirS, nirK and norB, norVW respectively. The conversion of nitrous oxide to nitrogen gas marks the final step and the enzymes responsible are nitrous oxide reductase and nosZ respectively [29].

### 1.6.1.2 Nitrate Ammonification / Assimilation



**Fig 1.4: Nitrate Ammonification and Assimilation Cycle**

Nitrate is broken down to nitrite, the key enzyme in this conversion is nitrate reductase and the genes involved are narGHI, napAB, nasA. This being followed by conversion of nitrite to ammonia.

A series of reactions involving reduction of inorganic nitrogen by microbes are essentially anaerobic pathways beginning with  $\text{NO}_3^-$  and ending with the production of  $\text{N}_2$  gas [30]. The availability of N oxides, nitrite ( $\text{NO}_2^-$ ) or nitrate ( $\text{NO}_3^-$ ) in the autotrophic nitrification pathway, are the key to the process of denitrification. Input of chemical fertilizers and organic soil matter mineralization are the vital sources of ammonium ( $\text{NH}_4^+$ ) in the environment. The production of nitrous oxide has been linked to the following biological processes, first under aerobic conditions during the process of nitrification of  $\text{NH}_4^+$  and second usually under anaerobic conditions coupled nitrification/denitrification pathway. The prevalence of suitable environmental conditions and formation of  $\text{NO}_3^-$  are associated with N oxides reduction by microbes, the suitable conditions comprise of low- or no- oxygen concentrations, and high soluble C content [31]. Respiratory denitrification is a bacterial respiratory process that involves the sequential reduction of nitrous oxides coupled with electron transport phosphorylation [32].

### 1.6.2 Anaerobic denitrification

A number of Gram-positive and gram-negative bacteria utilize inorganic nitrogen oxides as electron acceptors for respiratory growth under anaerobic conditions. This can result in decrease of nitrogen fertilizers from the soil. This denitrification process can be used for bioremediation of environmental contaminants in oxygen limiting environments [33]. For a long time, anaerobic denitrification was thought to be the only form of denitrification. It is performed as a type of respiration and reduces oxidized forms of nitrogen. In this thesis denitrification is carried out aerobically and measured by reduction in nitrate and production of nitrite and ammonium. The preference to nitrogen electron acceptors are as follows - nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), nitric oxide (NO), nitrous oxide ( $\text{N}_2\text{O}$ ) finally resulting in the production of dinitrogen ( $\text{N}_2$ ) completing the nitrogen cycle [34].

### 1.6.3 Aerobic denitrification

Conventional understanding of the denitrification process dictates that this process is necessarily anaerobic. However, research has shown that aerobic denitrification can be achieved through laboratory cultures. Though the process of denitrification is predominantly anaerobic, denitrifiers are taxonomically and physiologically diverse. Simultaneous consumption of  $\text{O}_2$  and  $\text{NO}_3^-$  as alternative terminal acceptors in respiration is not precluded in many bacteria [35]. Aerobic denitrifiers are known to carry out an aerobic respiratory process where nitrate is gradually converted to  $\text{N}_2$  with several intermediates ( $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$ ), using nitrate reductase (Nar or Nao), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) respectively. Various species isolated from activated sludge, wastewater and treatment plants have been shown to reduce nitrate or are involved in part of the process of the reduction of oxidised nitrogen species through to nitrogen gas in aerobic or partially aerobic conditions. These species include *Alcaligenes faecalis*, *Shinella zoogloeoides*, *Agrobacterium* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Comamonas* sp., *Paracoccus denitrificans*, *Klebsiella pneumonia*, *Pseudomonas stutzeri*, *Rhodococcus* sp. and *Citrobacter diversus* [36].

**Table 1: Strains of bacterial population capable of denitrification**

Species/Strain	Metabolism	References
<i>Pseudomonas denitrificans</i>	Facultative anaerobic	[36]
<i>Pseudomonas aeruginosa</i>	Facultative anaerobic	[39]
<i>Thiobacillus denitrificans</i>	Aerobic	[37]
<i>Micrococcus denitrificans</i>	Facultative anaerobic	[38]
<i>Paracoccus denitrificans</i>	Facultative anaerobic	[36, 39]
<i>Alcaligenes faecalis</i>	Aerobic	[36, 39, 40]
<i>Zoogloea sp.</i>	Aerobic	[36, 41]
<i>Shinella zoogloeoides</i>	Aerobic	[36, 39, 42]
<i>Agrobacterium sp.</i>	Aerobic	[36, 39, 43]
<i>Acinetobacter sp.</i>	Aerobic	[36, 39, 44]
<i>Pseudomonas sp.</i>	Aerobic	[36, 39, 45]
<i>Comamonas sp.</i>	Aerobic	[36, 39, 46]
<i>Klebsiella pneumonia</i>	Aerobic	[36, 39, 47]
<i>Pseudomonas stutzeri</i>	Aerobic	[48]
<i>Rhodococcus sp.</i>	Aerobic	[49]
<i>Citrobacter diversus</i>	Aerobic	[50]
<i>Mesorhizobium sp.</i>	Aerobic	[51]
<i>Halomonas campisalis</i>	Aerobic	[52]

<i>Marinobacter sp.</i>	Aerobic	[53]
<i>Rhodobacter sphaeroides</i>	Anaerobic	[54]
<i>Thauera mechemichensis</i>	Anaerobic	[55, 56]
<i>Comamonas denitrificans</i>	Anaerobic	[55]
<i>Thauera phenylacetica</i>	Anaerobic	[57]
<i>Thauera aminoaromatica</i>	Anaerobic	[57]

## 1.7 Denitrifying bacterial strains chosen for research

A great deal of diversity exists in bacteria capable of performing denitrification, this group comprises over 50 genera with over 125 different species and represent 10-15% of bacterial population in water, soil and sediment [58,59]. There are theories that state that denitrification and aerobic respiration rose at the same time from a common ancestor. This common ancestor is thought to be part of a purple photosynthetic bacterial group. This is contradictory to the other more commonly thought belief that the common ancestor was a nitrate-reducing organism. Denitrification seems to have risen at the same time as aerobic respiration; these seem to have evolved from a common ancestor in the purple photosynthetic bacterial group and not from nitrate-reducing organisms [60]. Denitrifying bacteria are facultative aerobic heterotrophs and are capable of utilizing oxygen and nitrate simultaneously as a terminal electron acceptor [58,59]. These denitrifying bacteria require an organic carbon source for heterotrophic denitrification [61], and utilize a broad range of inorganic and organic compounds as sources of carbon and energy [62].

The denitrifying bacteria used for this research were chosen because of the different routes mentioned in literature, to accomplish denitrification. The five strains were *Bacillus subtilis*, *Paracoccus denitrificans*, *Acidovorax* spp., *Pseudomonas stutzeri* and *Paenibacillus macerans* are discussed below in more detail -

### 1.7.1 *Paracoccus denitrificans*

*Paracoccus denitrificans*, previously known as *Micrococcus denitrificans* is one of the gram negative bacteria found in soil known for its degradative properties and bioremediating denitrifying properties [63]. This bacteria was first discovered by a Dutch microbiologist in 1908 [64] and belongs to the alpha subdivision of proteobacteria. The metabolically flexible ability of the bacteria to live in both aerobic and anaerobic conditions also make it thrive in extremophilic environments. Typically, the bacteria is observed to be a non-motile coccoid, however rod shaped cells in young cultures [65] are often observed. It also drives energy from a long list of organic and inorganic compounds with a few exceptions of mineral medium supplemented with methanol or sodium acetate [66]. The denitrification pathway employed by

*Paracoccus denitrificans* includes conversion of nitrates to nitrogen gas via intermediates that include nitrites, nitrous oxides and other volatile gases [67].

Denitrification of *Paracoccus denitrificans* includes respiratory reduction of nitrates and nitrites into molecular nitrogen (N<sub>2</sub>) through nitrogen oxygen reduction. Proteome analysis of the PD1222 species of bacteria has revealed several molybdenum, copper and iron-dependent pathways depending on different forms of nitrogen which are denitrified by the bacteria [68]. On transcriptomic level, transition of the bacteria from aerobic to anaerobic phase leads to a 15- to 45- fold increase of denitrifying enzyme mRNAs. Several intermediates are produced during the transition phase, however on successful transition, dinitrogen was observed to be the bacteria's main product [69]. Given bacteria's characterization as an extremophile, studies were performed to study its denitrifying pathways at suboptimal pH (pH of 6.8). It was observed that the denitrifying activity at suboptimal pH reduced significantly, which might lead to further studies on identifying optimal pH, temperature and conditions that can help in optimized denitrification with minimal byproducts.

### 1.7.2 *Pseudomonas stutzeri*

*Pseudomonas stutzeri* is an aerobic, gram negative denitrifying bacteria [70]. Isolating and identifying this bacteria in the laboratory environment was challenging due to their unusual shape and consistency before its genome was characterized. However, simpler and cheaper ways to classify bacteria are still undergoing research. This bacteria forms colonies and are typically hard, dry and coherent [71]. The pathway it employs for nitrate reduction involves the conversion of nitrates to nitrogen gas via intermediates that include nitrites, nitrous oxides and other volatile gases [67]. Studies have shown to prove simultaneous nitrification and denitrification being carried out by this strain and it also carries out denitrification in the presence of high oxygen levels.

Many strains of *Pseudomonas stutzeri* are known to possess transformative properties [71]. Transformative properties in bacteria refer to the ability of bacteria to take up foreign genetic material such as DNA, or also release them into the environment. This ability combined with its denitrification capability has interested scientists to consider this organism as a model organism for studying denitrification.



### 1.7.3 *Bacillus subtilis*

*Bacillus subtilis* is a remarkably diverse Gram positive, rod shaped facultative aerobe and is capable of vegetation in diverse environments [72], [73]. It is one of the vastly studied bacteria due to its abundant presence in the environment, ease of culturing with optimal growth temperature of 37°C [74], and the effortlessness in culturing them. During nitrate respiration *Bacillus subtilis* reduces nitrate via nitrite to ammonia and hence is known as an ammonifying strain of bacteria [75].

Several studies have been performed with various *Bacillus subtilis* strains to identify optimal temperatures for denitrification. The B3 process (Bio Best Bacillus), a Korean patented process which is used for wastewater treatment to remove nitrogen, phosphorus and other inorganic compounds appeared to be the most economical way to do it [76]. There is an environmental advantage to using this bacteria given its presence in the natural ecosystem and plays an important role in the nitrogen cycle.

### 1.7.4 *Acidovorax spp.*

This group of species belong to the genus Proteobacteria, and is currently known to constitute less than 20 species. They are observed within nature in sludge and aqueous environments, with straight to slightly curved rod shape. Those include *Acidovorax facilis*, *Acidovorax delafieldii*, *Acidovorax temperans*, *Acidovorax defluvii*, *Acidovorax avenae*, *Acidovorax konjaci*, *Acidovorax anthurii* and *Acidovorax valerianellae* and *Acidovorax caeni sp. nov.* [77]. All the species within the genus are facultative anaerobes and mostly chemoorganotrophs. Lithoautotrophic characteristics within the genus can be observed with *A. facilis* and *A. delafieldii* [78], [79]. Similar to other denitrifying bacteria, the genes that are responsible for denitrification are nirS and nosZ genes.

### 1.7.5 *Paenibacillus macerans*

*Paenibacillus macerans* previously classified and known as *Bacillus macerans* is regarded as a Gram positive facultative anaerobic bacterium, belonging to the genus *Paenibacillus* [80], [81]. Its optimal growth habitat is 30°C. Unlike many other bacteria mentioned in the text, *P. macerans* happens to be a diazotroph, meaning that they fix molecular nitrogen present in the environment into ammonia. Nitrate and nitrite respiration by this strain of bacteria produces ammonia and is hence known to take the route of nitrate ammonification rather than denitrification.

On successful completion of denitrification of nitrates and nitrites to molecular nitrogen using some of the bacteria mentioned above, there is economic advantage to convert this into usable forms of nitrogen such as ammonia. *P. macerans* and other diazotrophs can help in this process.

## PART TWO: METHANOTROPHS

### **1.8 Live Methanotrophs to Methanotrophic biomass**

One of the primary objectives of the research was to study denitrification of bacteria in detail. We started this by culturing carefully chosen bacteria to identify the optimal concentration of nitrate, nitrite and ammonia transformed, and produced across different growth phases of bacteria. The objective of this thesis is to enhance and supplement the current research carried out in the field of denitrification given the increase of nitrates and nitrites observed in water bodies that disturbs the natural balance of ecosystem. Scalability is an important limiting factor between research and industrial settings, and hence our secondary aim was to identify the concentration of carbon and energy source required that yielded the optimal growth of bacteria. Methanotrophs, both in live and biomass forms can serve as a source of carbon and energy to support the growth of denitrifying bacteria, but also facilitate denitrification. Yeast extract, one of the carbon-rich sources, and a commonly used culturing medium for bacteria was used to test this hypothesis. Two reasons drove the motivation behind choosing methanotrophs to test this hypothesis. First, for mass and industrial-scale production, a bioreactor can be used to culture both denitrifiers and methanotrophs. Hence, methanotrophs were isolated from an enriched culture and an experiment was designed to test the hypothesis on a pilot scale. The water burdened with nitrate can be pumped through the reactor and back to the reservoir through a filter. Second, the methanotrophic biomass served as an alternative to the idea of bacterial biomass present in the aquifer being used by the denitrifying bacteria to survive. One such approach was to use biomass from aquifer grown methanotrophs (methane-oxidizing bacteria) to stimulate denitrification. In this Master's research, I undertook laboratory-based research to support this ESR/CAPE research by demonstrating aerobic denitrification and testing if denitrifiers can utilize methanotrophic biomass, as a carbon and energy source.

## 1.9 Methanotrophs

Methanotrophs are prokaryotic organisms that have the ability to metabolize methane. This characteristic makes methanotroph an interesting bacterial species to study due to the need of reduction of greenhouse gases. They are fundamental players in the process of methane cycle as they oxidize concentrations of methane in the atmosphere [82]. Methanotrophs are known to be obligate aerobes which use methane as their sole source of carbon and energy [83]. This misconception of methanotrophs being obligately methanotrophic and not being able to utilize single bond carbon compounds was discarded with the isolation and study on some members of the genera *Methylocella*, *Methylocystis* and *Methylocapsa* [84]. These methanotrophs that tend to be aerobic in nature are unique bacteria that can metabolize not only methane but also some single carbon compounds as sole sources for carbon and energy [85].

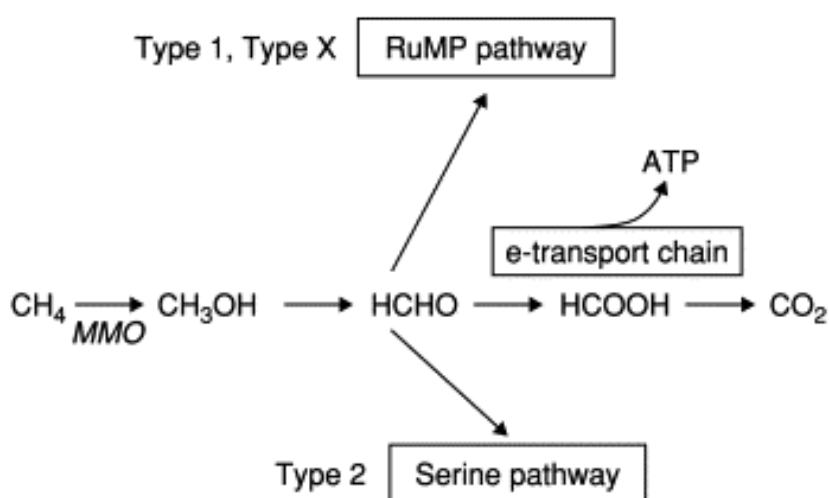
Aerobic methanotrophs can be found in various ecosystems, usually extreme conditions such as terrestrial, aquatic and marine. They are usually found on surfaces due to the availability of oxygen, which can be used as the final electron acceptor while methane can be used as a carbon and energy source. These bacteria have the inert ability to adapt to conditions, especially when they are present in conditions with high methane concentration. They can oxidize methane in the environment, if oxygen is not available [86].

Whittenbury and colleagues work in the 1960s led to segregation of methanotrophs into three categories based on source of carbon and energy and other physiological and morphological characteristics [82, 87]. Type I and Type X belong to the family *Methylococcaceae* and are a part of Gammaproteobacteria (class), which uses the RuMP pathway. The other kind type II belongs to the family *Methylocystaceae*, is a part of Alphaproteobacteria (class) and utilises the serine pathway [83].

Given the recent rise in greenhouse gases emitted into the environment such as methane, studying prokaryotes such as methanotrophs can help understand the natural mechanisms and metabolic pathways that can be harnessed to reset the balance in the environment. Methanotrophs are known to naturally oxidize very low concentrations of methane (~ 2 parts per million by volume - ppmv) found in the atmosphere, which is not high enough with respect to the emissions created by humans.

## 1.10 Pathways for methane/carbon metabolism

The initial step is a common step in the metabolism pathway for all methanotrophs, conversion of methane to methanol by the enzyme MMO (methane monooxygenase) and then the transformation of methanol to formaldehyde [88]. Although the manner in which carbon metabolism takes place is unique to each methanotroph family. The ribulose monophosphate pathway (RuMP) is employed by type I and type X methanotrophs. On the other hand type II methanotrophs utilize the serine pathway.



**Fig 1.5: Pathways employed by methanotrophic bacteria for carbon assimilation.** Type 1 and type X employ the RuMP pathway whereas type 2 employ the serine pathway [89]

Recent studies have focused on understanding the metabolic pathways that lead to the oxidation of methane. Monooxygenase enzyme, a protein present in methanotrophs, helps the bacteria in the oxidation of methane. The essential cofactors of the enzyme are either copper or iron, thus an important component for designing culturing medium for methanotrophs. Metalloproteomics of the monooxygenase enzyme led to the discovery of Csp1 (Copper storage protein 1), a critical protein that helps in the storage of copper by binding to multiple cysteine residues present in the protein [90]. Two forms of methane monooxygenase are known - soluble methane monooxygenase (sMMO), and particulate methane monooxygenase (pMMO) - a membrane bound enzyme. On further studies of these enzymes, particulate methane monooxygenase (pmoA) was identified as a functional biomarker for aerobic methanotrophs [91].



## CHAPTER TWO

### Materials and Methods

## **2.1 General bacterial culture methods**

### **2.1.1 Media Preparation**

Media were prepared for culturing denitrifiers and methanotrophs. All media were sterilized via autoclave at 121°C at 15 psi for at least 20 minutes. The media were made sure to be homogenous, and the pH for all media were adjusted to pH 7.0 before sterilization unless stated otherwise.

#### **2.1.1.1 Nutrient Broth**

13g of nutrient broth powder obtained from Sigma-Aldrich was added to 800 ml of Milli-Q water. The medium was shaken well before the volume was brought up to 1L and then sterilized.

#### **2.1.1.2 Nutrient Agar plates**

15g of nutrient agar powder and 13g of nutrient broth powder obtained from Sigma-Aldrich was added to 800ml of Milli-Q water. The medium was shaken well before the volume was brought up to 1L and then sterilized. About 15-20ml of autoclaved media was poured on bacterial culture plates in Laminar Air Flow (LAF), and left to solidify. After solidification, they were then stored and refrigerated in 4°C.

#### **2.1.1.3 Lysogeny Broth (LB medium)**

15.5g of BD Difco™ Luria Broth Base, Miller obtained from Fisher Scientific was added to 800ml of Milli-Q water. The dehydrated media was then stirred on a shaker at 300 rpm at 25°C for the components to dissolve and create a homogenous mixture. The medium was then brought up to 1L and then autoclaved.



#### 2.1.1.4 Trace Element and Fe.EDTA composition

The below components were used to create a supplementary media which is used in further and used as an essential component for other medias – such as denitrification and modified denitrification media.

##### Trace Elements:

ZnSO <sub>4</sub> .7 H <sub>2</sub> O	0.44mg	(= 0.1 mg Zn)
CuSO <sub>4</sub> .5 H <sub>2</sub> O	0.20mg	(= 0.05 mg Cu)
MnCl <sub>4</sub> .4H <sub>2</sub> O	0.19mg	
Na <sub>2</sub> MoO <sub>4</sub> .2 H <sub>2</sub> O	0.06mg	(= 0.024 mg Mo)
H <sub>3</sub> BO <sub>3</sub>	0.10mg	(= 0.02 mg B)
CoCl <sub>2</sub> .6 H <sub>2</sub> O	0.08 mg	(= 0.02 mg Co)

The weights of the above components are given per ml of milli-Q water. Stocks of 100ml were prepared ahead of time.

##### Fe.EDTA:

FeSO <sub>4</sub> .7 H <sub>2</sub> O	1.54g
Na <sub>2</sub> EDTA	2.06g

The weights of the above components are given per ml of milli-Q water. Stocks of 100ml were prepared ahead of time.

#### 2.1.1.5 Denitrification Media

Sodium citrate	5g
Potassium nitrate	1g
Potassium dihydrogen phosphate	1g
Potassium hydrogen phosphate	0.5g
Magnesium sulfate heptahydrate	0.2g
Trace elements	2ml
Fe.EDTA	3ml
Potassium iodide	0.12g

The above components were dissolved in approximately 800ml of Milli-Q water and stirred at 300rpm before the pH was calibrated and then the volume was brought to 1L with Milli-Q water. The medium was then poured into Erlenmeyer flasks and autoclaved.

#### 2.1.1.6 Modified Denitrification Media

Yeast extract	0.1% - 0.4% (w/v)*
Nitrate	1mg – 11mg, 1g **
Potassium dihydrogen phosphate	1g
Potassium hydrogen phosphate	0.5g
Magnesium sulfate heptahydrate	0.2g
Trace elements	2ml
Fe.EDTA	3ml
Potassium iodide	0.12g

\* The concentration of yeast extract was varied – 0.1, 0.2, 0.3, 0.4% w/v for identifying optimal concentration that harbors bacterial growth.

\*\* Potassium Nitrate ( $\text{KNO}_3$ ) stock solution were created such that the nitrate concentration varied from 1mg, 3mg, 5mg, 7mg, 9mg, 11mg, and 1g

The above components were dissolved in approximately 800ml of Milli-Q water and stirred at 300rpm before the pH was calibrated and then the volume was brought to 1L with Milli-Q water. The medium was then poured into Erlenmeyer flasks and autoclaved.

#### 2.1.1.7 Denitrification Media with methanotroph biomass

Methanotroph biomass	0.5% (v/v)*
Nitrate	7mg **
Potassium dihydrogen phosphate	1g
Potassium hydrogen phosphate	0.5g
Magnesium sulfate heptahydrate	0.2g
Trace elements	2ml
Fe.EDTA	3ml
Potassium iodide	0.12g

\* The methanotroph biomass obtained externally was concentrated and lysed, of which 0.5% v/v was added as an energy source

\*\* Potassium Nitrate (KNO<sub>3</sub>) stock solution were created such that the nitrate concentration was 7mg.

The above components were dissolved in approximately 800ml of Milli-Q water and stirred at 300rpm before the pH was calibrated and then the volume was brought to 1L with Milli-Q water. The medium was then poured into Erlenmeyer flasks and autoclaved.

#### 2.1.2 Colony isolation and culturing

Bacterial cultures were handled in Laminar Air Flow (LAF) at all times as a part of general culturing practice.

Frozen culture samples of denitrifying bacteria were obtained from ESR (Institute of Environmental Science and Research) and thawed via gentle agitation at room temperature. A loopful of defrosted bacterial suspension was aseptically transferred to solid medium (Nutrient Agar – Sigma Aldrich) and incubated statically at their optimum incubation temperature (see below). Grown bacterial colonies were then isolated for each denitrifying bacteria and culture purity was verified by observing colony morphology under a microscope.

<b>Denitrifying bacterial strains</b>	<b>Optimum growth temperature</b>
<i>Acidovorax spp.</i>	28°C
<i>Bacillus subtilis</i>	37°C
<i>Pseudomonas stutzeri</i>	37°C
<i>Paracoccus denitrificans</i>	28°C
<i>Paenibacillus macerans</i>	30°C

### 2.1.3 Sub-culturing of cultures

Single colonies isolated were passaged at least three times by streaking a loopful of bacterial culture on nutrient agar plates (Nutrient Agar – Sigma Aldrich) to ensure culture purity and to allow bacteria to adapt to the common energy source. Axenic cultures were then further grown in Nutrient broth (Nutrient Broth – Sigma Aldrich) and grown aerobically at 200 r.p.m up to an OD of 0.80. The grown axenic cultures were then (1) directly used for further research discussed in this thesis, and (2) cryopreserved for future studies. The bacterial strains were sub-cultured onto new sterile Nutrient Agar plates to maintain an active stock of bacteria.

### 2.1.4 Cryopreservation of cultures

To generate cryopreserved stocks, pure cultures inoculated in Nutrient Broth which were grown aerobically (as discussed in the previous section) were used. The culture was mixed with 50% glycerol and added to microcentrifuge tubes. They were then successfully stored at -80°C.

### 2.1.5 Growth measurements via Optical Density (O.D.)

As experiments for growth measurements were carried out, 1-2 ml of culture suspension was taken at an interval of 30 min in a Laminar Air Flow. All experiments are performed in triplicates. The OD of the suspension was measured using a UV spectrophotometer at 600nm where the sterile medium used for culturing was used as a blank.

At the end of the experiment, graph of OD (representing cell count) against time was plotted to study various phases of bacterial growth.

## **2.2 Bacterial culture in various culture media**

### **2.2.1 Bacterial culture in LB and denitrification media**

Growth of denitrifying bacteria was studied by culturing them in Lysogeny Broth and denitrification medium. Bacterial growth was measured using Optical Density as discussed in Section 2.1.5.

During experiments studying growth curve, rapid and substantial increases in pH after 4-6 hours of growth in denitrification media was observed. This is due to the use of sodium citrate as a carbon source, where the metabolic byproducts produced cause an increase in Ph. As a result of this experiment, the ratio of xx to xx was changed in the denitrification medium and used for the subsequent experiments.

It was noted during routine cultivation of *P. macerans*, that if cultures were inoculated with active liquid culture, the bacteria had a tendency to form aggregates. To avoid this, the loopful colony isolated was first thoroughly mixed in a microcentrifuge tube with the medium before inoculating in the liquid medium.

### **2.2.2 Bacterial culture in Modified Denitrification Media**

To reflect the objectives of the thesis, it was critical to see if the denitrifying bacteria selected had the ability to grow in a complex medium such as methanotroph biomass. The bacteria were cultured in modified denitrification medium discussed above. The modified denitrification medium includes yeast extract as a carbon and energy source which is a complex media – similar to the methanotroph biomass.

Concentrations of yeast extract and nitrate concentration were varied across different experiments to study optimal concentrations of bacterial growth. Bacterial growth was measured using Optical Density as discussed in Section 2.1.5. On identification of optimal concentrations of yeast extract and nitrate, denitrification capabilities of bacteria i.e. reduction in nitrate and increase in nitrite and ammonia were measured using analytical techniques discussed in Section 2.3.

### 2.2.3 Bacterial culture in denitrification media with methanotroph biomass and live methanotroph culture

After success in growth of denitrifying bacteria using a complex media – yeast extract, the bacteria were then cultured in denitrification media with methanotroph biomass, and also with live methanotroph culture. The growth was measured using optical density (OD) as discussed in Section 2.1.5. The denitrification capabilities of bacteria were measured using the analytical techniques discussed in Section 2.3 to understand the optimal concentrations at which the bacteria most optimally is observed to denitrify.

## **2.3 Analytical measures for determining nitrate, nitrite and ammonia concentration**

The reagents and powder pillows used to measure the concentrations of nitrate, nitrite and ammonia were acquired from HACH. The instrument used to measure the concentration was DR900 colorimeter and the settings were modified to measure the increase or decrease in concentrations of nitrate, nitrite and ammonia respectively. The sample cell used for this purpose was of Product ID#2401906.

Standards for nitrate, nitrite and ammonia

1. For nitrate, standard solutions of 0.010, 0.050, 0.150, 0.300mg/L nitrate ( $\text{NO}_3^-$ -N) was prepared from nitrate standard solution (1000mg/L) obtained from Sigma Aldrich.
2. For nitrite, standard solutions of 2, 4, 8 and 16 mg/L nitrite ( $\text{NO}_3^-$ -N) was prepared from nitrite standard solution (1000 mg/L) obtained from Sigma Aldrich.
3. For ammonia, standard solutions of 0.10, 0.20, 0.40mg/L ammonia ( $\text{NH}_3^+$ -N) were prepared from ammonia standard solution (1000 mg/L) obtained from Sigma Aldrich.

All the prepared standard solutions were filtered through 0.2  $\mu\text{m}$  plasma membrane.

### **2.3.1 Measurement of nitrate concentration**

The cadmium reduction reaction was employed to determine the nitrate concentration. Nitrate reagent powder pillows NitraVer® 5 from HACH (Product No. 2106169) was used for testing. The range for measurement of nitrate concentration ranged from 0.3mg/L to 30mg/L.

The nitrate was measured as per the manufacturer's instructions, and the steps are as follows –

1. Two sample cells were filled with 10ml of liquid - one for blank, and one will for testing
2. NitraVer 5 Nitrate Reagent Powder pillow was added to the solution to be tested for reaction
3. The mixture is shared vigorously for 1 minute for the powder to dissolve in the solution
4. The mixture is then let to react for 5 minutes and then measured by DR900 colorimeter machine after being calibrated with blank.

### 2.3.2 Measurement of nitrite concentration

USEPA diazotization was employed for measurement of nitrite concentration. NitriVer 3 Reagent Powder was used for testing.

The nitrite was measured as per the manufacturer's instructions, and the steps are as follows –

1. Two sample cells are filled with 10ml of liquid - one for blank, and one for testing
2. NitriVer 3 Reagent Powder pillow was added to the solution to be tested for reaction
3. The solution is then swirled and inspected for any color change. Pink color indicates presence of nitrite.
4. The concentration of nitrite was then measured by DR 900 colorimeter machine after being calibrated with blank.

### 2.3.3 Measurement of ammonia concentration

Salicylate method is employed for detecting the concentration of ammonia. The range for measurement of ammonia concentration ranged from 0.01 to 0.50 mg/L ammonia nitrogen. Ammonia reagent powder pillows, ammonia salicylate and ammonia cyanurate from HACH, (Product No. 2668000) was used for testing.

The ammonia was measured as per the manufacturer's instructions, the steps are as follows –

1. One sample cell is filled with 10ml of liquid to be tested, and another sample cell with 10ml of deionized water
2. Ammonia Salicylate powder is added into each cell and the mixture was shaken for the reagent to dissolve and incubate for 3 minutes
3. After the timer expires, Ammonia cyanurate is added into each cell and the mixture was shaken for the reagent to dissolve and incubate for 15 minutes
4. A change in color to green indicates presence of ammonia-nitrogen
5. The concentration of nitrite is then measured by DR 900 colorimeter machine after being calibrated with blank using the 8155 program.



## **2.4 Isolation and species identification of live methanotrophs**

### **2.4.1 Methanotrophic biomass extraction**

Live methanotroph culture was obtained from GNS science, New Zealand. It was known that these are methanotrophic bacteria, but the species were not identified. Following steps were performed for extraction of methanotroph biomass, which was then used in the denitrifying medium with methanotrophs (discussed in Section 2.1.1.7)

1. The culture was grown in anaerobic conditions till it reached an OD of 0.85
2. The culture was then centrifuged at 5000rpm for 10 minutes
3. Most of the supernatant was aspirated and the bacteria was boiled for less than 2 minutes
4. The culture was then sonicated using SonyPrep 150 disintegrator for ultrasonic disintegration at power 5.
5. Methanotrophic biomass was either used right away to create medium, which was later autoclaved or stored at 4°C.

### **2.4.2 Isolation of enriched methanotrophs**

It was also unknown if there was only one or multiple methanotrophic bacteria. For species identification, the culture was serially diluted by extinction in denitrification medium such and grown in anaerobic conditions i.e. air in the serially diluted tubes was replaced with methane and incubated for 24-48 hours until colonies grew. The colonies were then isolated and species identification was performed as discussed below.

### 2.4.3 Genome sequencing

On the growth of colonies, the methanotrophs were further cultured in anaerobic conditions for culture purity. Two primary steps were performed to extract DNA and sequence the genome for identification of species

1. Soil DNA isolation using Nucleospin Soil (Takara Cat #740780.50)
2. DNA Clean and Concentrator Kit (DCC) (Zymo Research Cat #D4033)

The following steps were performed for DNA isolation

1. About 300mg of live methanotrophic culture was added to Nucleospin Bead Tube A
2. 700 µl of Lysis Buffer SL1 was added
3. 150 µl of Enhancer SX was added and the cap was closed
4. The samples were vortex for 5 min at room 20°C
5. Contaminants were precipitated by centrifuging at 11,000 x g for 2 min
6. The supernatant was separated and stored in a separate microcentrifuge tube
7. 150 µl of Enhancer SL3 was added to the tube and vortexed for 5s
8. Incubated for 5 mins at 0°C
9. Centrifuged at 11,000 x g for 1 min
10. NucleoSpin inhibitor Removal column was placed on the top of the microcentrifuge tube
11. 700 µl of supernatant separated in Step 6 was added to the inhibitor removal column
12. The tube(s) were centrifuged at 11,000 x g for 1 min
13. 250 µl of Buffer SB was added and the tube was vortexed for 5 s
14. Nucleospin soil column was placed on the top of a collection tube
15. 550 µl of sample was added to this column
16. Solution was centrifuged at 11,000 x g for 1 min
17. Flow-through was discarded and the remaining sample was again added to the column
18. Solution was re-centrifuged at 11,000 x g for 1 min
19. Flow-through was discarded and column was put into the collection tube
20. The silica membrane was washed with 550 µl Buffer SB and 550 µl Buffer SW1. The buffer solution was centrifuged at 11,000 x g for 30 s
21. Finally, 700 µl of Buffer SW was added, the tube was vortexed and re-centrifuged at 11,000 x g for 30 s

22. The silica membrane is dried by centrifugation of the tube at 11,000g for 2 min
23. 50 µl of Buffer SE was added to the column for elution of DNA and incubated at room temperature for 1 min
24. The buffer solution was centrifuged at 11,000 x g for 30s. This buffer solution includes DNA

The following steps were performed for DNA purification and concentration –

All centrifugation steps were performed at 11,000 x g

1. 200 µl of DNA binding buffer was added 100 µl of DNA solution (obtained from above steps)
2. The mixture was transferred to ZymoSpin column in a microcentrifuge tube
3. The tube was centrifuged for 30s and the flow through was discarded
4. 200 µl of DNA wash buffer was added and the tube was re-centrifuged. The step was repeated once more
5. 200 µl of DNA elution buffer was added to column matrix, incubated at room temperature for 1 min and centrifuged for 30 s to elute DNA
6. The elution now included ultra-pure DNA

The concentration of ultra-pure DNA eluted using the above steps was checked using NanoDrop and was found to be 148.4µg/µL

#### 2.4.4 Species Identification

These 16s ribosomal RNA regions within bacteria are highly conserved across species, and hence very helpful for phylogenetic analysis. The rRNA is a component of 30S small subunit of a prokaryotic ribosome.

To identify methanotroph species, the 16s rRNA sequence in the genome can be amplified and sequenced. Universal Primers were used for sequencing the targeted region –

#	Primer Name	Primer Sequence (5' – 3')
1	9F	GAGTTTGATCITIGCTCAG
2	1492R	TACGGYTACCTTGTTACGACTT

The region was amplified in a PCR and sent out at the sequencing center.

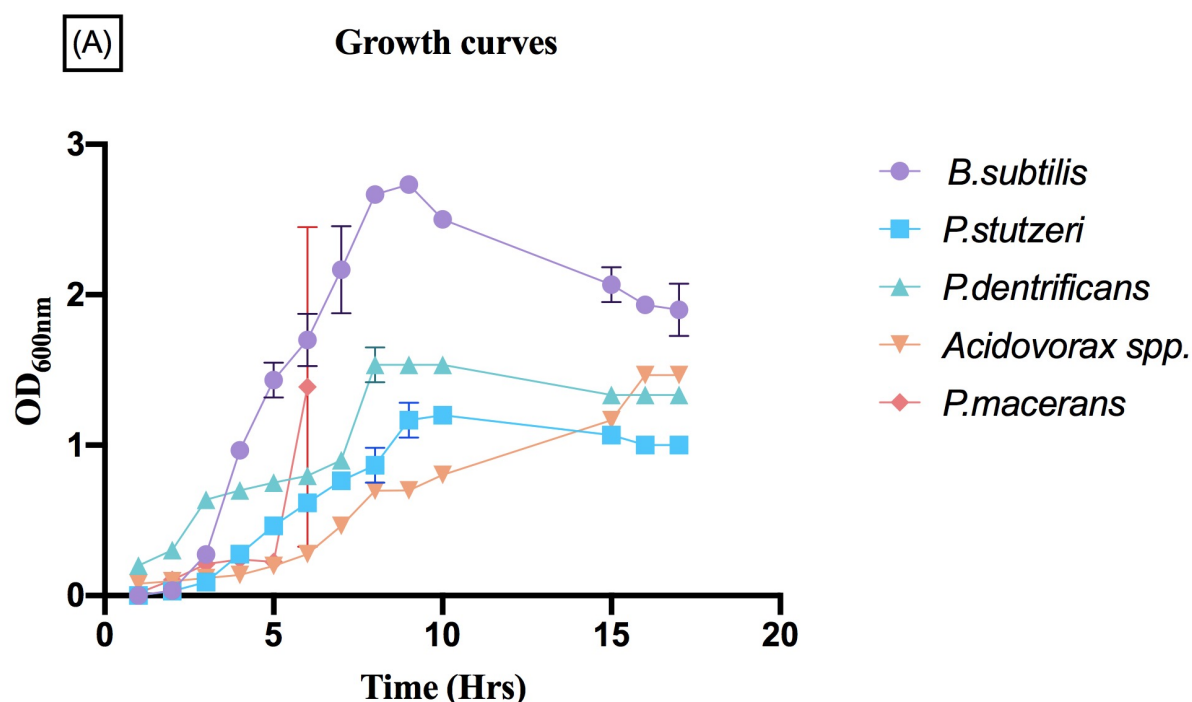


## CHAPTER THREE

### Results

### 3.1 Standard growth curves for all denitrifying strains cultivated in Lysogeny Broth (LB)

Standard growth curves plotted for all strains were cultivated in Lysogeny Broth (LB medium). These growth curves were used as a standard for these strains. These cultures were diluted to obtain graphs establishing a relationship between OD, cell per ml and time.



**Fig 3.1: Bacterial growth curves plotted for denitrifying bacteria in LB**

(A) *B. subtilis*, *P. stutzeri*, *P. denitrificans*, *Acidovorax spp.* and *P. macerans*<sup>3</sup>.

#### Observation

All the strains of bacteria except *P. macerans* were observed to cross an OD of 1.5. Some strains were observed to reach OD as high as 3.0. *P. macerans* was observed to form aggregates and a change in concentration of inoculum levels did not make a difference. On further troubleshooting about the stage at which *P. macerans* forms clumps, it was observed

<sup>3</sup> Incubation temperature- *B. subtilis* 37°C, *P. stutzeri* 37°C, *P. denitrificans* 30°C, *Acidovorax spp.* 30°C. and *P. macerans* 30°C.

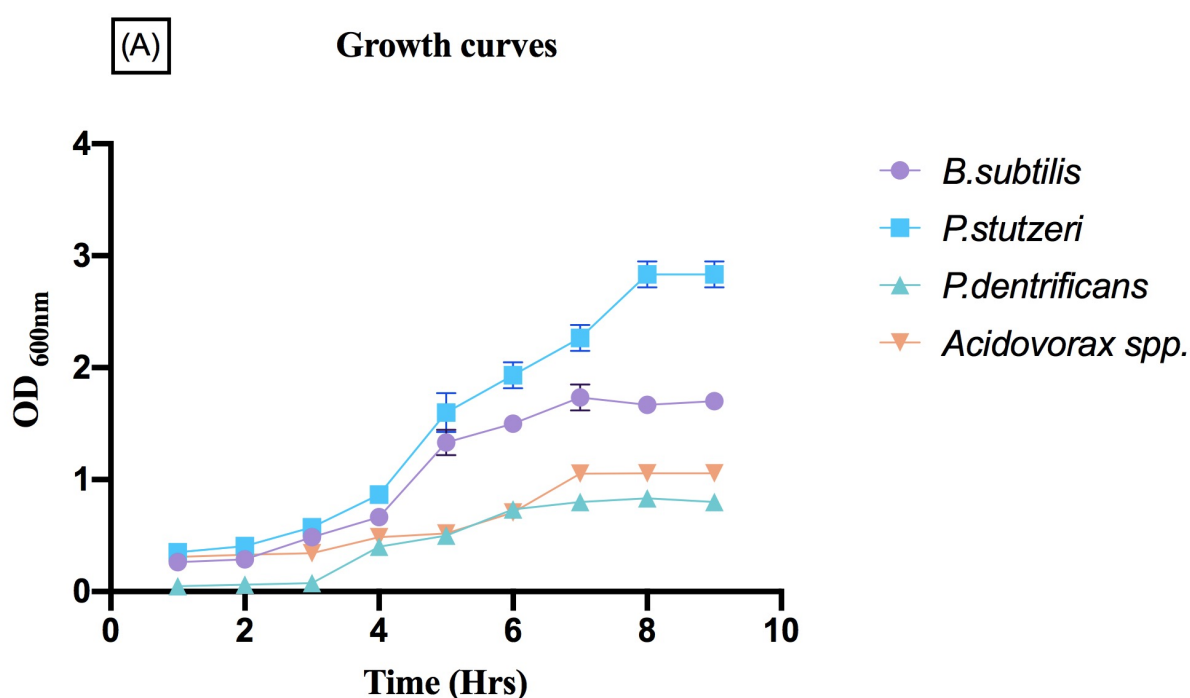
Shaking incubator speed- 200rpm, volume of media- 100ml, volume of inoculant- 1ml

that the bacteria formed clumps no later than entering the exponential phase, beginning with small aggregate which grew with time.

The pH was monitored throughout the growth cycle showing no major shifts in pH. The culture was alive and in abundance when observed under the microscope.

### 3.2 Standard growth curves for all denitrifying strains cultivated in Denitrifying Media

Bacterial strains in study were cultured in denitrification media and growth curves were plotted. The objective was to compare the difference in growth curves of bacterial species across LB medium and denitrification medium, which would aid in learning about the changes in growth pattern.



**Fig 3.2: Bacterial growth curves plotted for denitrifying bacteria in denitrifying media**

(A) *B. subtilis*, *P. stutzeri*, *P. denitrificans*, *Acidovorax spp.* and *P. macerans*.

#### Interpretation

XY statistical analysis which uses the nonlinear regression curve fitting was performed for the exponential (Malthusian) growth phase. It implied lower doubling time in comparison to the

growth in LB. Among all the bacteria tested, *P. macerans* was the only strain that did not grow in the denitrification media and was observed to form similar clumps as it formed in LB.

### 3.2.1 Cell counts

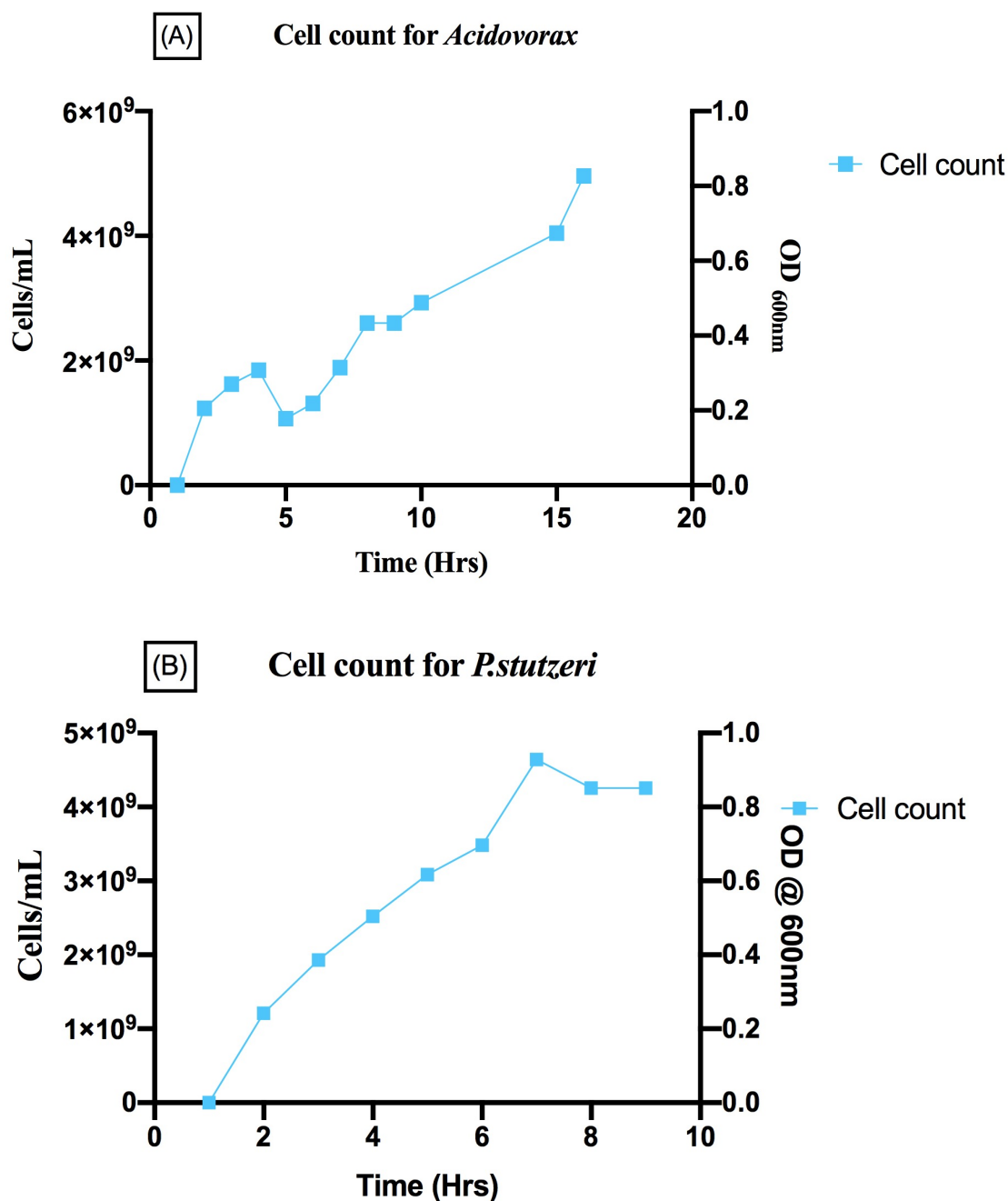


Fig 3.3: Cell counts for (A) *Acidovorax* spp. and (B) *P. stutzeri*



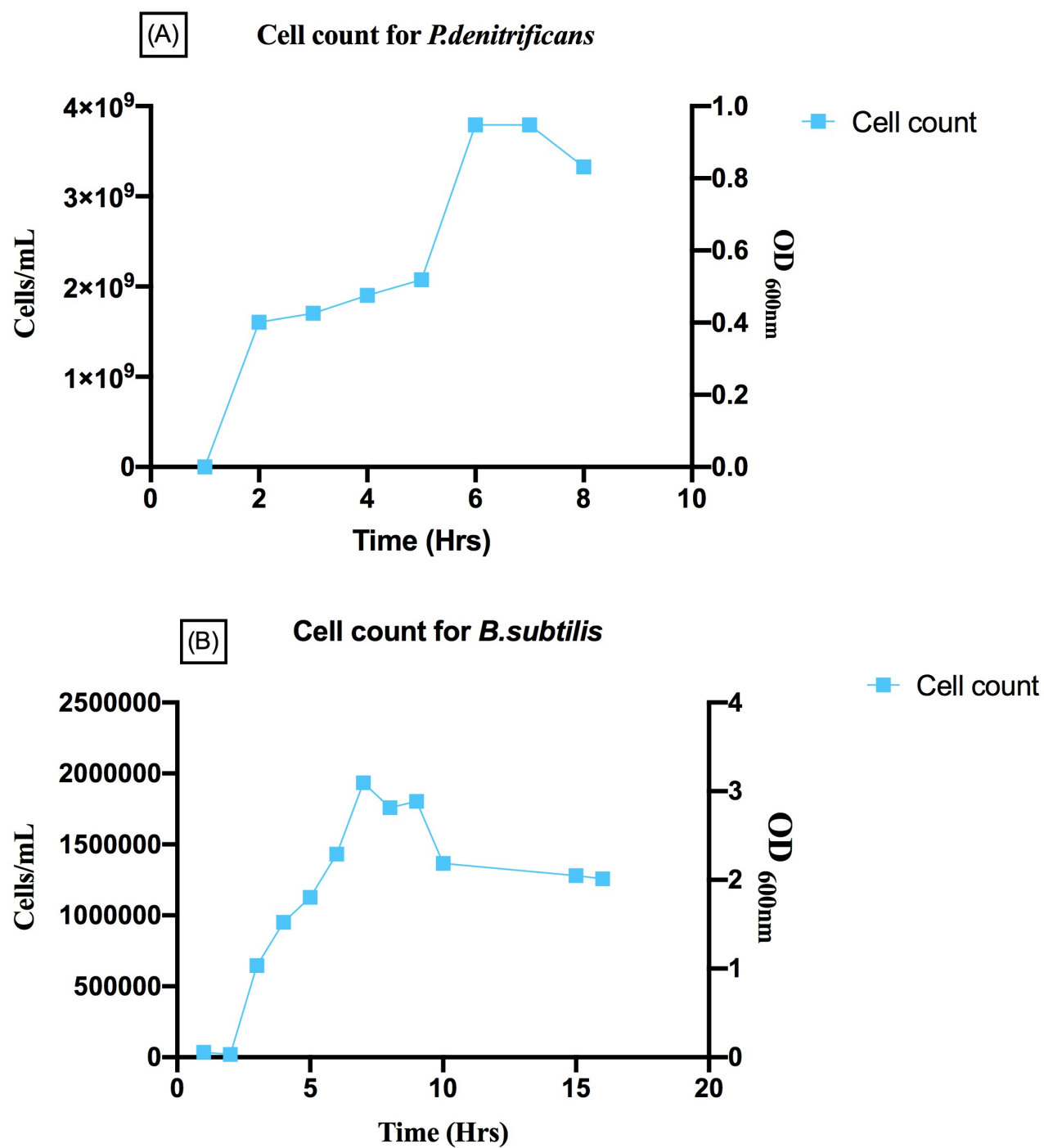
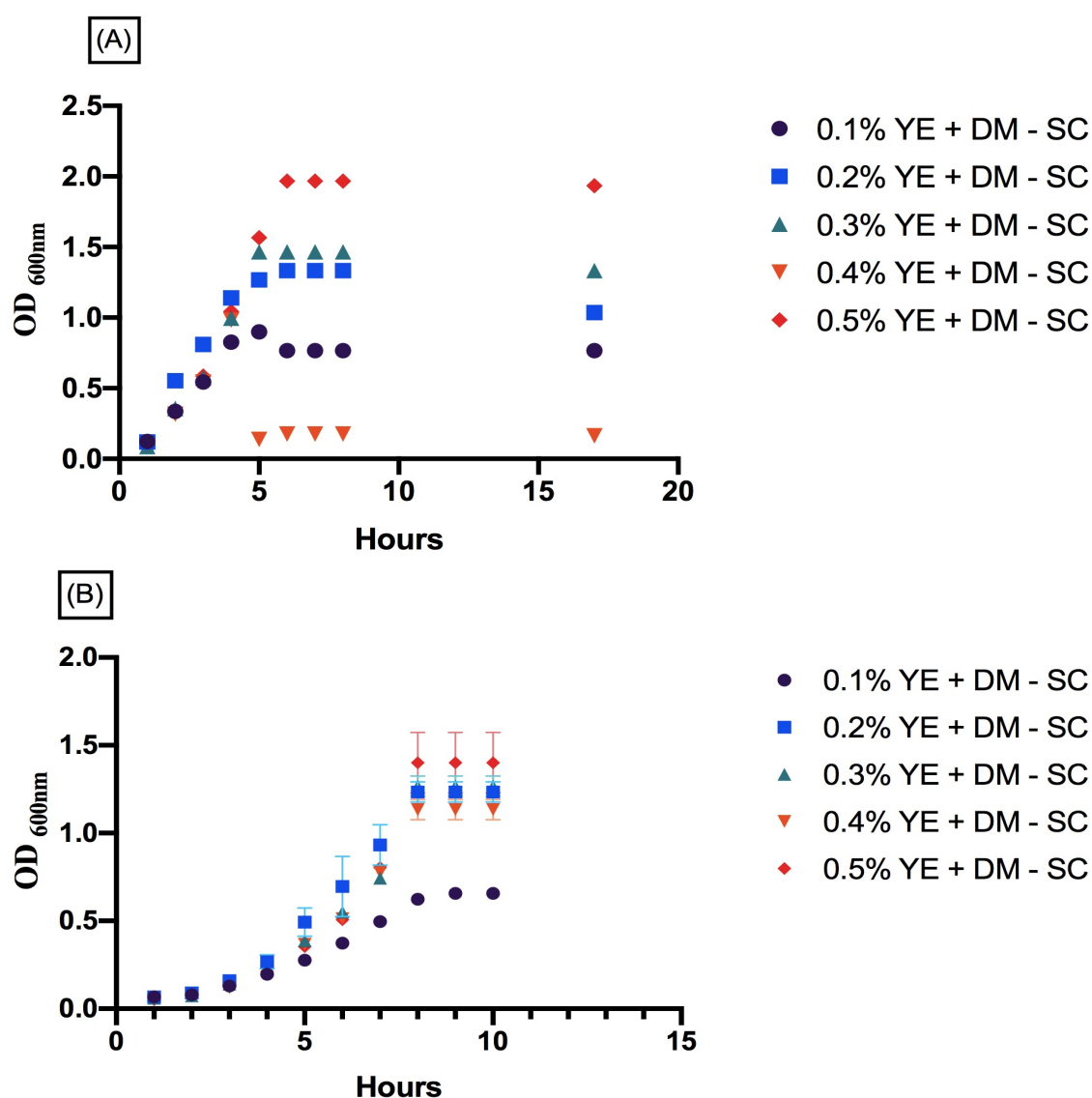


Fig 3.4: Cell counts for (A) *P. denitrificans* and (B) *B. subtilis*

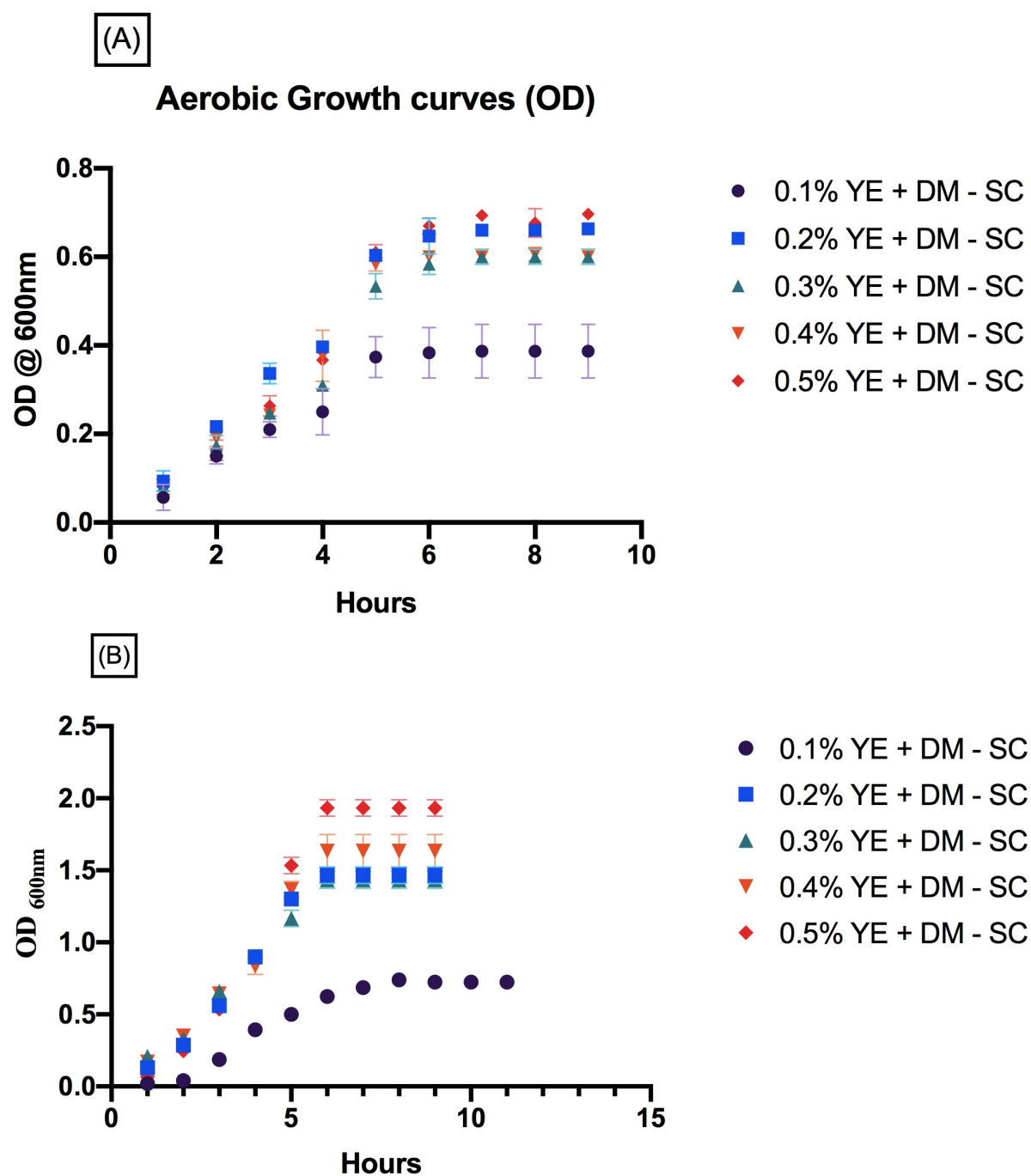
### 3.3 Identification of optimal yeast extract concentration in modified denitrifying media

Growth curves were plotted using two denitrification media - one with yeast extract, and one with sodium citrate (modified denitrification media) as the carbon and energy source. The concentration of yeast extract was varied in the following experiments to identify the optimal concentration at which denitrifying bacteria reduce nitrates.



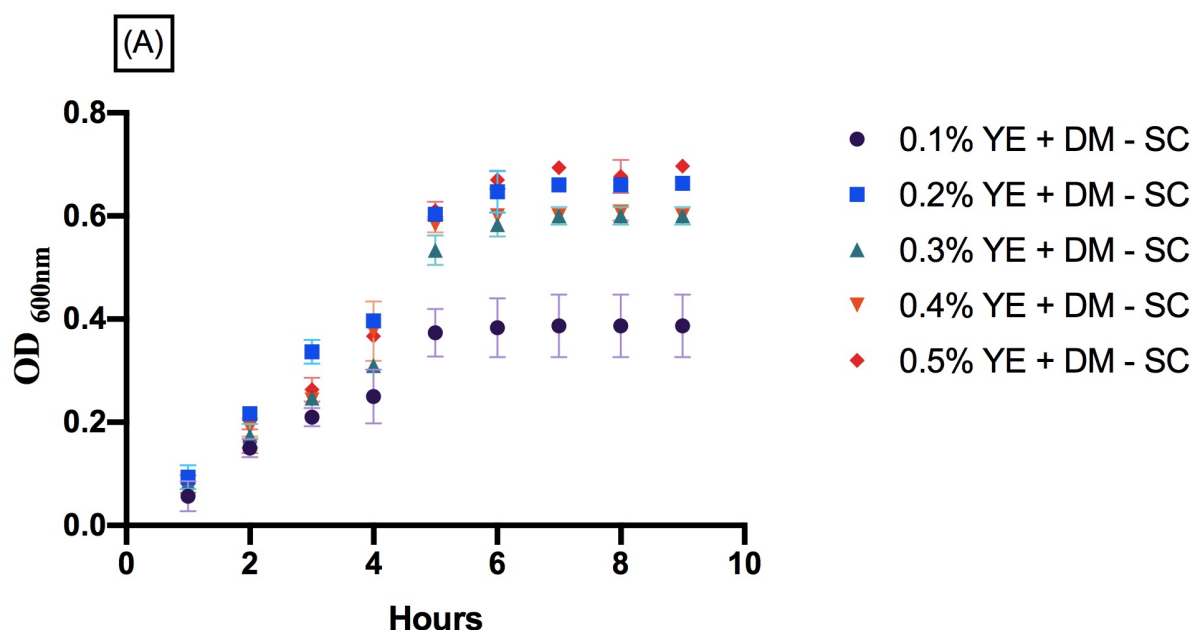
**Fig 3.5:** Bacterial growth curves plotted at varying concentrations of yeast extract (A) *Acidovorax* spp. and (B) *P. denitrificans*<sup>4</sup>.

<sup>4</sup> Incubation temperature- *B.subtilis* 37°C, *P.stutzeri* 37°C, *P.denitrificans* 30°C, *Acidovorax* spp 30°C. and *P.macerans* 30°C.



**Fig 3.6: Bacterial growth curves plotted at varying concentrations of yeast extract**  
 (A) *P. macerans* and (B) *B. subtilis*

Shaking incubator speed- 200rpm, volume of media- 100ml, volume of inoculant- 1ml



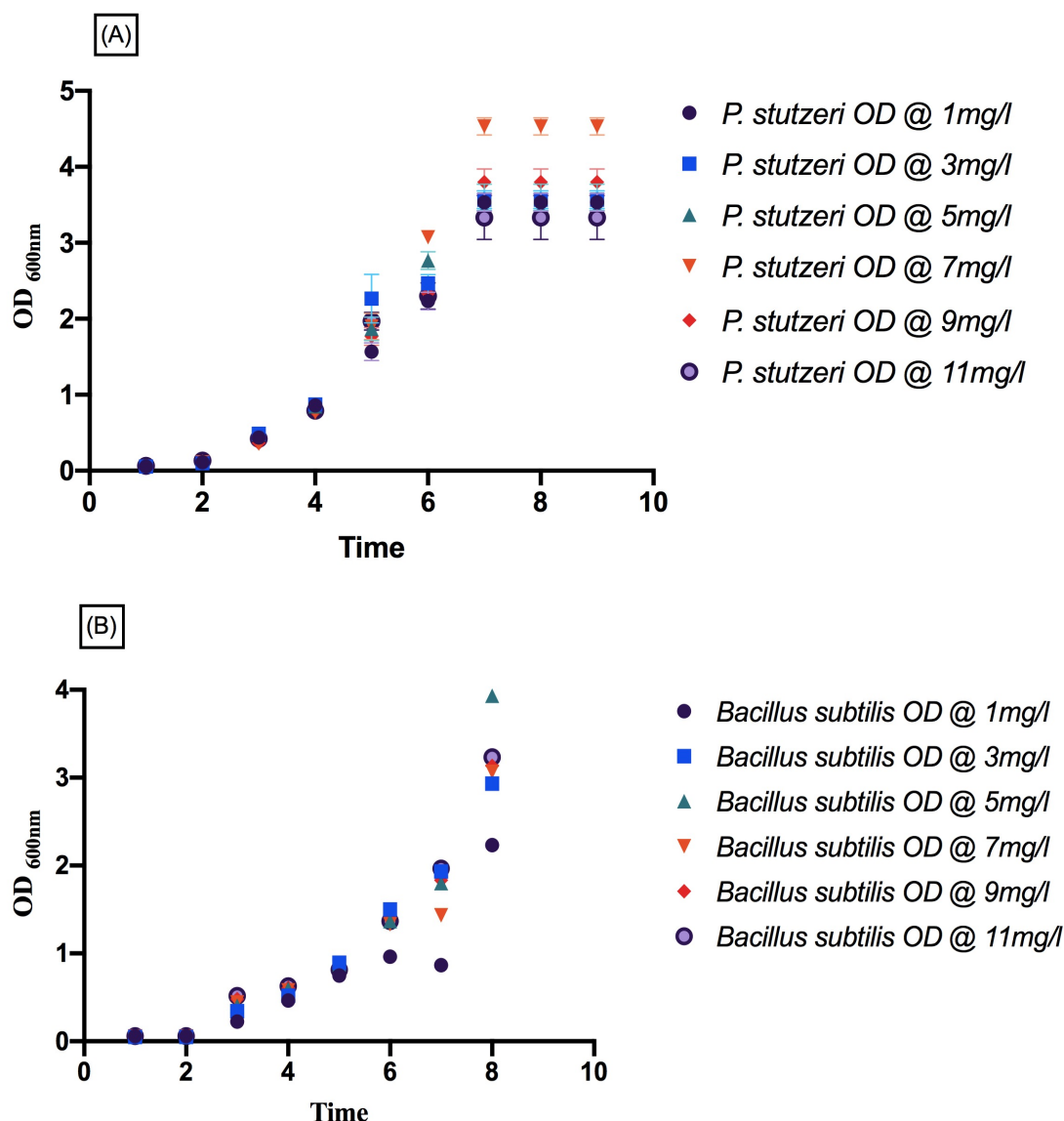
**Fig 3.7:** Bacterial growth curves plotted at varying concentrations of yeast extract  
(A) *P. stutzeri*.

### Interpretation

These bacterial growth curves represent growth of bacterial species in study across varying concentrations of yeast extract. Yeast extract can serve as the optimal growth medium for denitrifying bacteria. XY statistical analysis performed on the data displayed in the graphs showed no significant results that would favor one concentration over the other. Similar to previous experiments where growth is measured as OD, for these sets of graphs OD was chosen as the parameter to measure good growth. Subsequently, 0.5% concentration of yeast extract was chosen as it resulted in the highest value of OD. In all the succeeding experiments, 0.5% yeast extract was used for experimental studies.

### 3.4 Identification of optimal nitrate concentration in modified denitrifying media

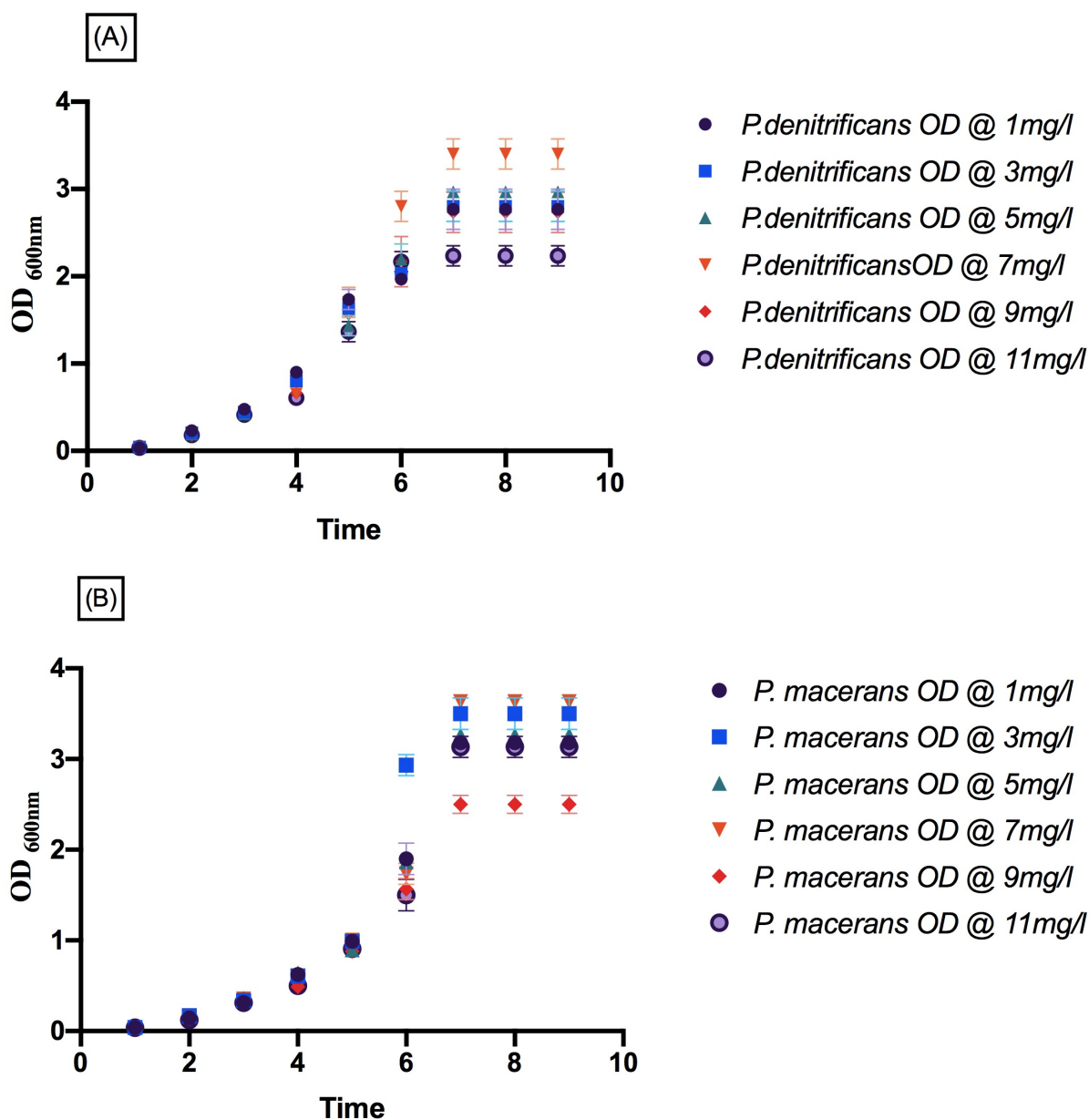
The concentration of nitrate was varied in the following experiments to identify the optimal concentration at which denitrifying bacteria reduce nitrates. Growth curves served as medium to identify optimal concentration that best supported growth and reduction of nitrate



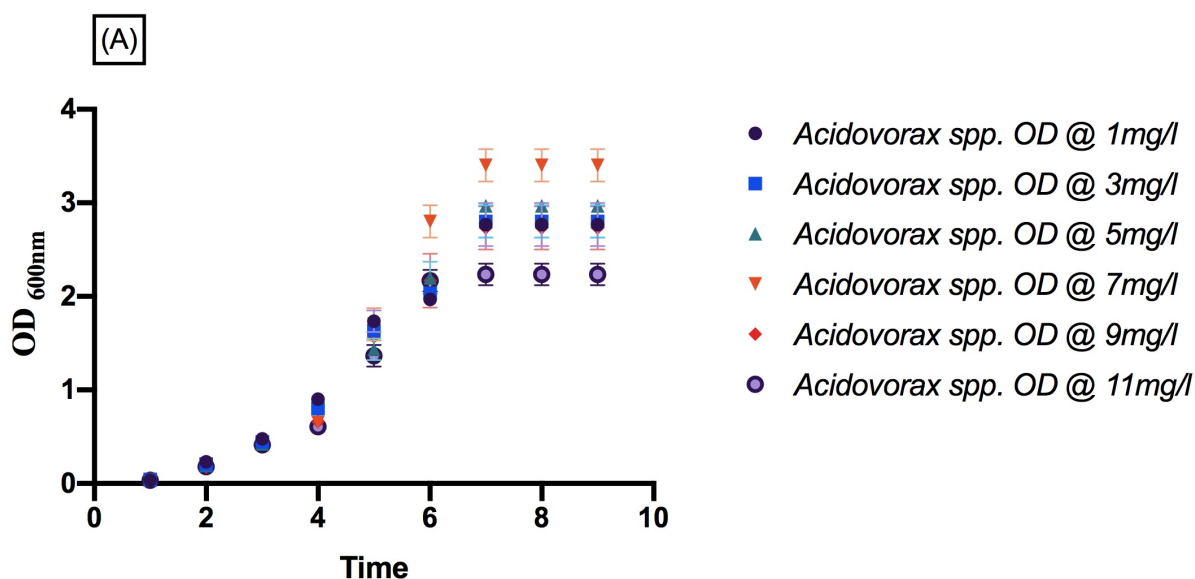
**Fig 3.8: Bacterial growth curves plotted at varying concentrations of nitrate ( $\text{NO}_3^-$ ) with 0.5% yeast extract in the growth medium of (A) *P. stutzeri* and (B) *B. subtilis*<sup>5</sup>.**

<sup>5</sup> Incubation temperature - *B. subtilis* 37°C, *P. stutzeri* 37°C, *P. denitrificans* 30°C, *Acidovorax* spp. 30°C. and *P. macerans* 30°C.

Shaking incubator speed- 200rpm, volume of media- 100ml, volume of inoculant- 1ml



**Fig 3.9:** Bacterial growth curves plotted at varying concentrations of nitrate ( $\text{NO}_3^-$ ) with 0.5% yeast extract in the growth medium of (A) *P. denitrificans*. and (B) *P. macerans*.



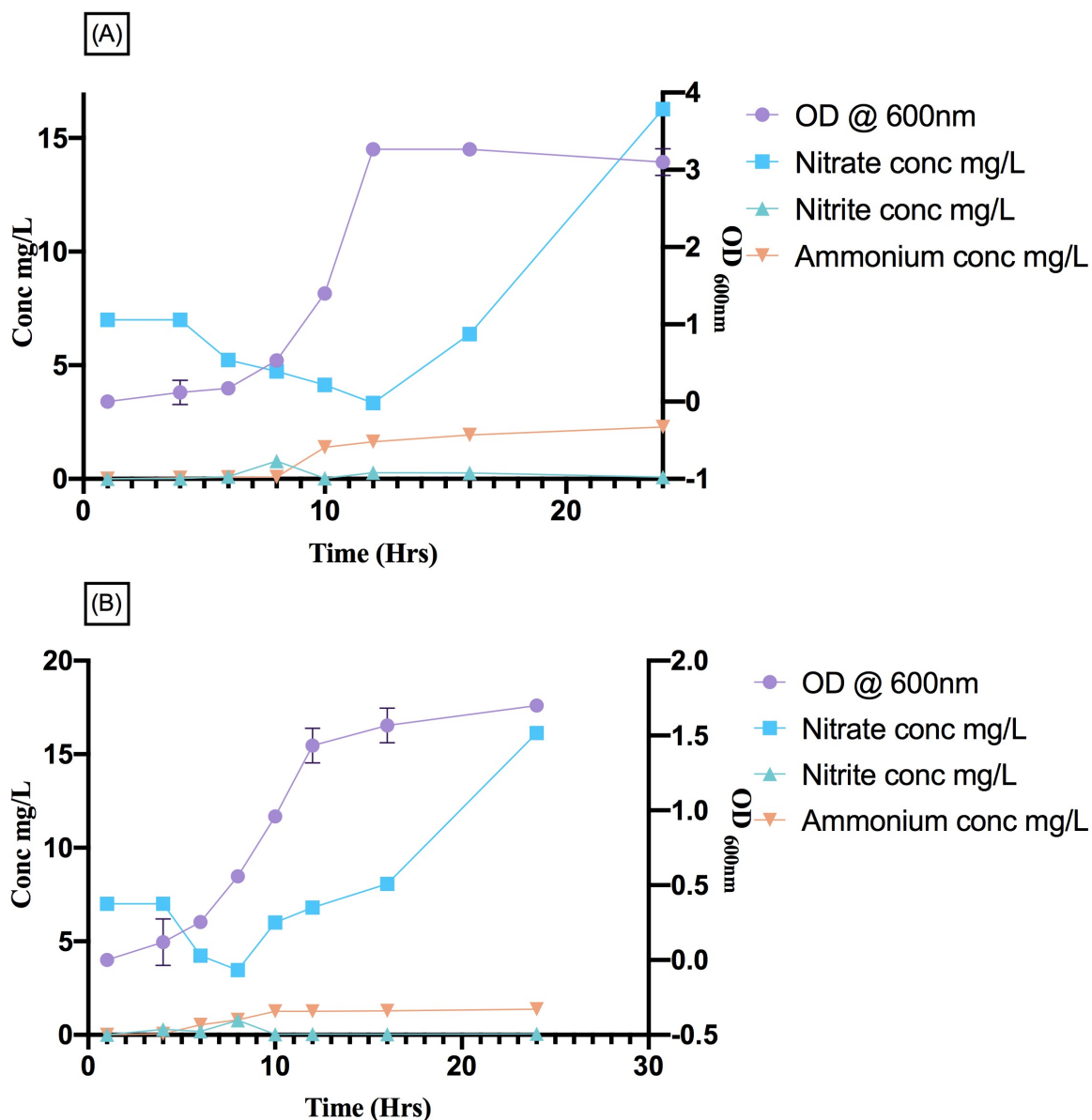
**Fig 3.10: Bacterial growth curves plotted at varying concentrations of nitrate ( $\text{NO}_3^-$ ) with 0.5% yeast extract in the growth medium of (A) *Acidovorax* spp.**

### Observation

Growth curves were plotted for experiments conducted in a denitrification medium with 0.5% yeast extract and varying concentration of nitrate, ranging from 1mg/L to 11mg/L. Up to 3mg/L of nitrate is known to be naturally found in the environment such as water reservoirs. Concentrations above 5mg/L are a result of anthropogenic activities. The graphs clearly show that the concentration of nitrate at 7mg/L with 0.5% yeast extract being optimal for growth of these strains. Thus, for future experiments, 7mg/L was chosen as the preferred nitrate concentration for the following experiments.

### 3.5 Change in concentration of nitrate, nitrite and ammonia over time

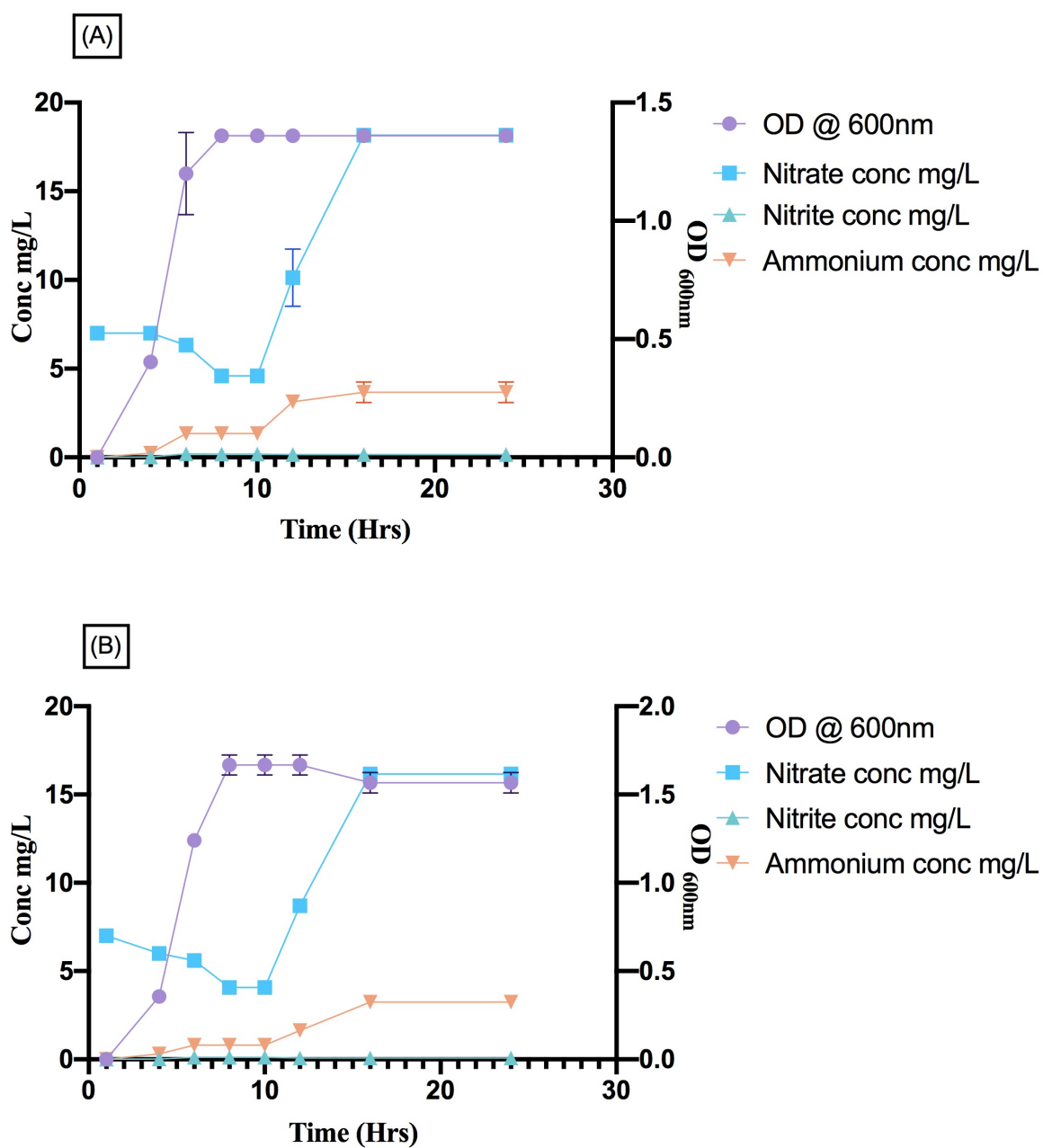
The method opted to measure denitrification was by measuring the reduction in concentration of nitrate and increase in concentration of nitrite and ammonia. These were plotted for all the strains against OD over a period of time



**Fig 3.11: Change in concentration of nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), ammonia ( $\text{NH}_3^+$ ) and OD over time (A) *B. subtilis* (B) *P. stutzeri*<sup>6</sup>.**

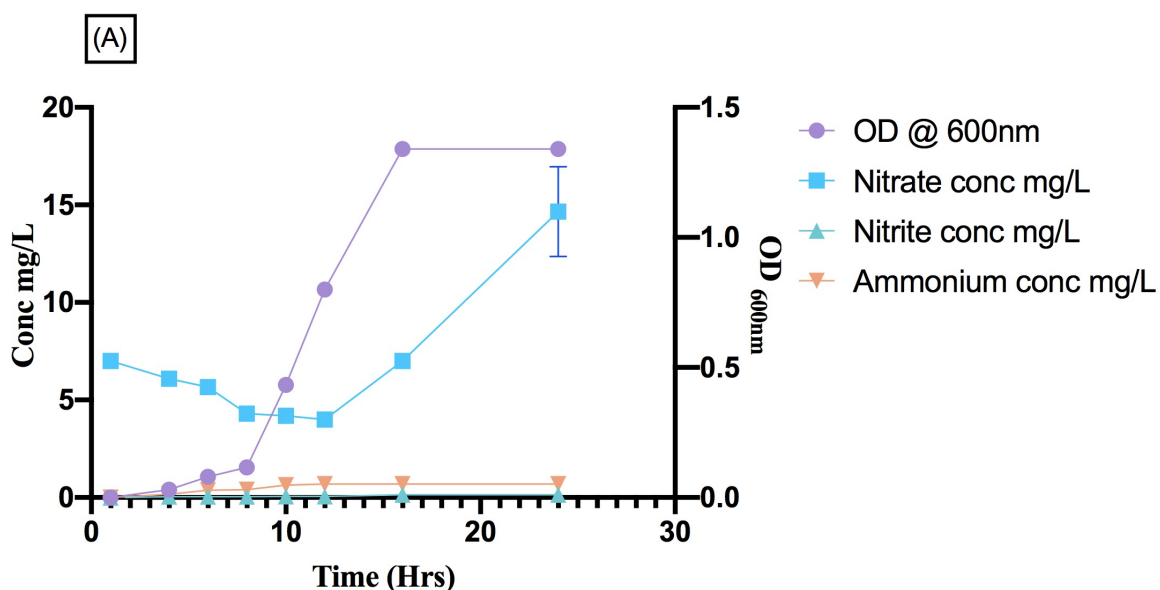
<sup>6</sup> Incubation temperature- *B. subtilis* 37°C, *P. stutzeri* 37°C, *P. denitrificans* 30°C, *Acidovorax spp* 30°C. and *P. macerans* 30°C.





**Fig 3.12:** Change in concentration of nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), ammonia ( $\text{NH}_3^+$ ) and OD over time (A) *Acidovorax* spp. and (B) *P. denitrificans*.

Shaking incubator speed- 200rpm, volume of media- 100ml, volume of inoculant- 1ml



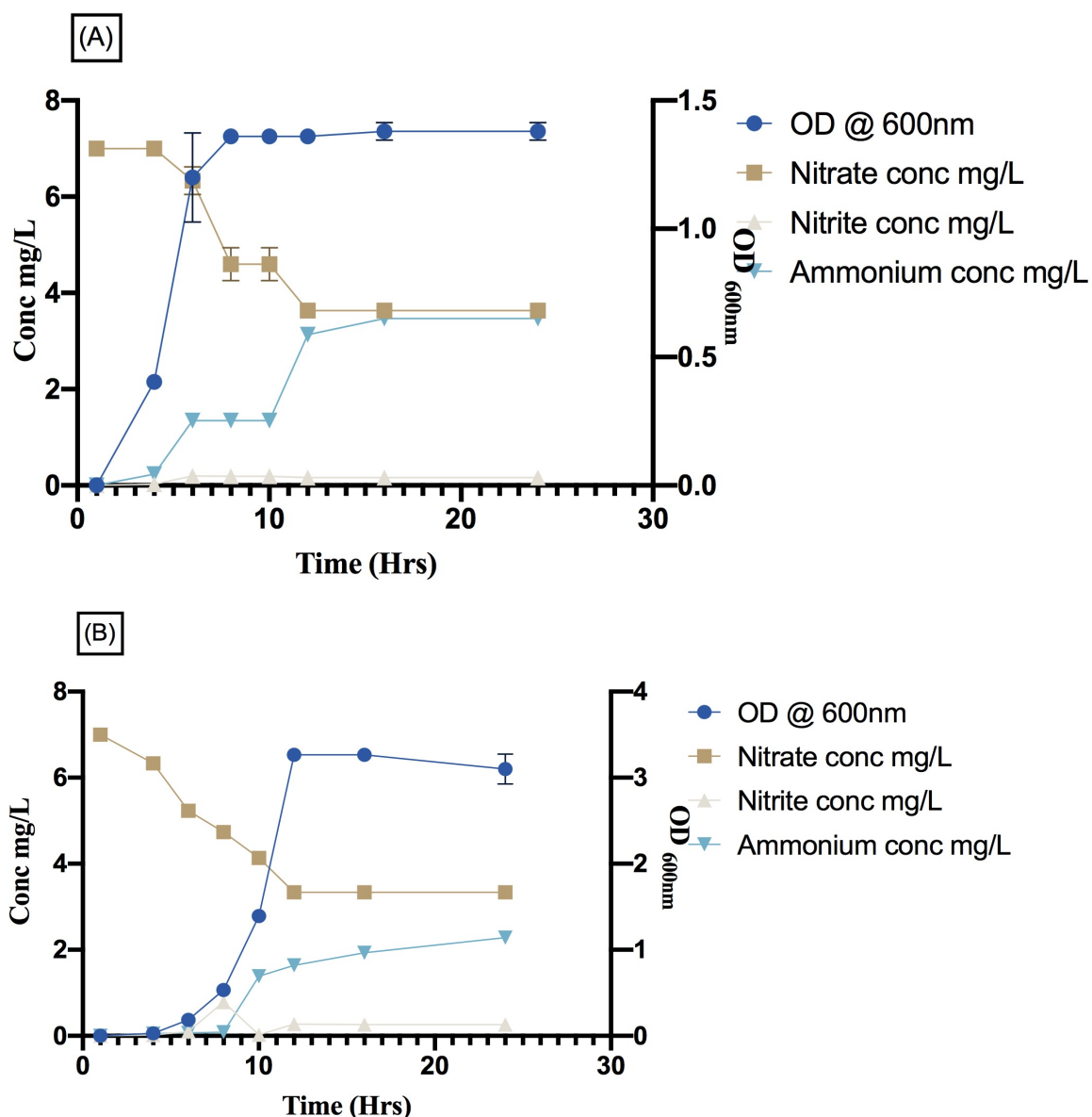
**Fig 3.13: Change in concentration of nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), ammonia ( $\text{NH}_3^+$ ) and OD over time (A) *P. macerans*.**

### Observations

These graphs were plotted to track the change in concentration of three main products namely nitrate, nitrite and ammonia, involved in the process of denitrification. The overall nitrate concentration remained constant at 7mg/L for nearly 4 hours (lag phase). A decrease in concentration of nitrate was observed post 4 hours (exponential phase) along with rise in concentration of nitrite and ammonia. There was an abrupt and significant increase in nitrate at a late exponential phase. To rule out yeast extract being the reason for this sudden rise in nitrate the following experiments were performed.

### 3.6 Change in concentration of nitrate, nitrite and ammonia with denitrification media

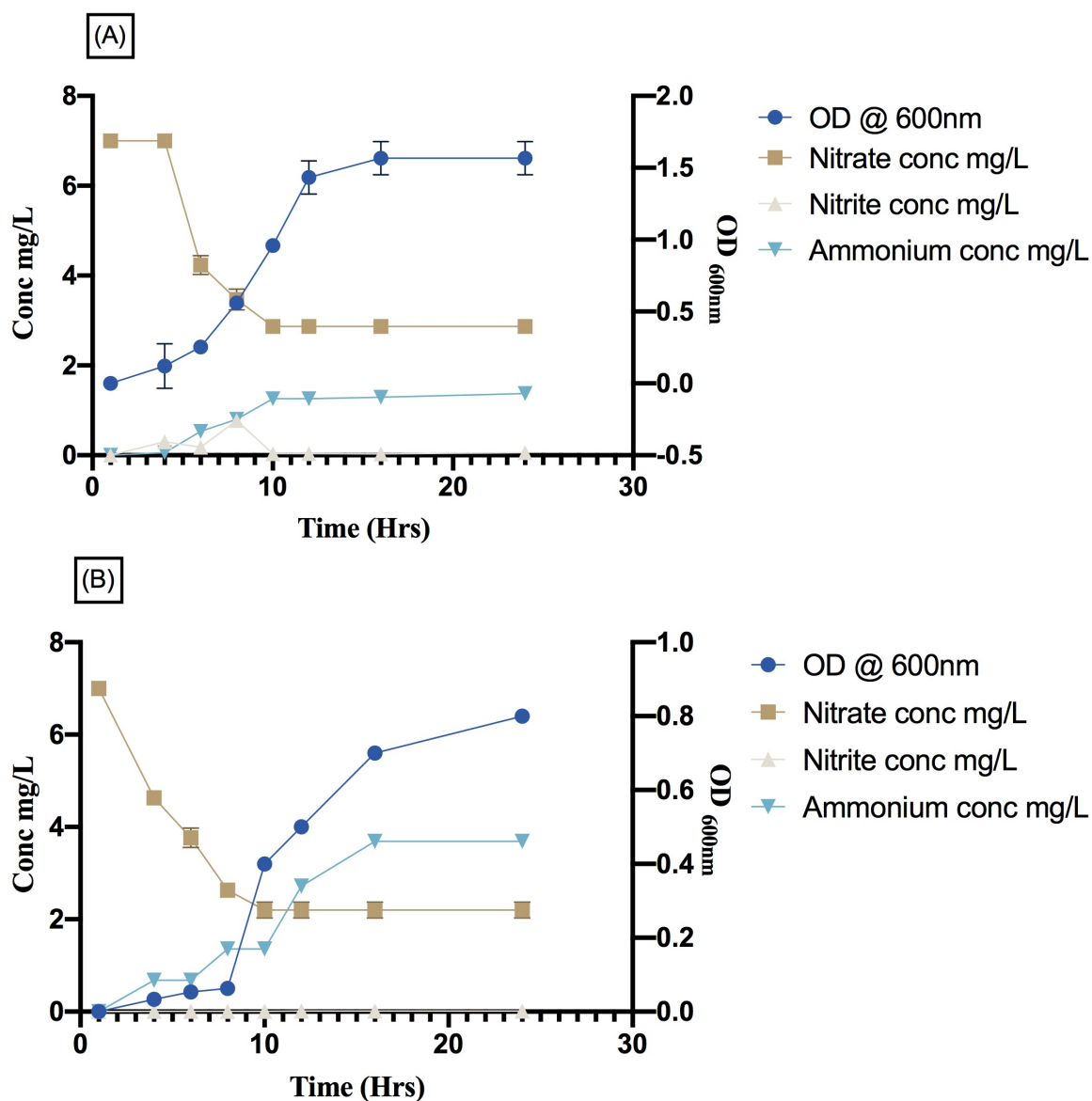
#### 3.6.1 Sodium citrate used as carbon/energy source in denitrification media



**Fig 3.14: Change in concentration of nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), ammonia ( $\text{NH}_3^+$ ) and OD over time (A) *Acidovorax* spp. (B) *B. subtilis*<sup>7</sup>.**

<sup>7</sup> Incubation temperature- *B. subtilis* 37°C, *P. stutzeri* 37°C, *P. denitrificans* 30°C, *Acidovorax* spp 30°C. and *P. macerans* 30°C.

Shaking incubator speed- 200rpm, volume of media- 100ml, volume of inoculant- 1ml

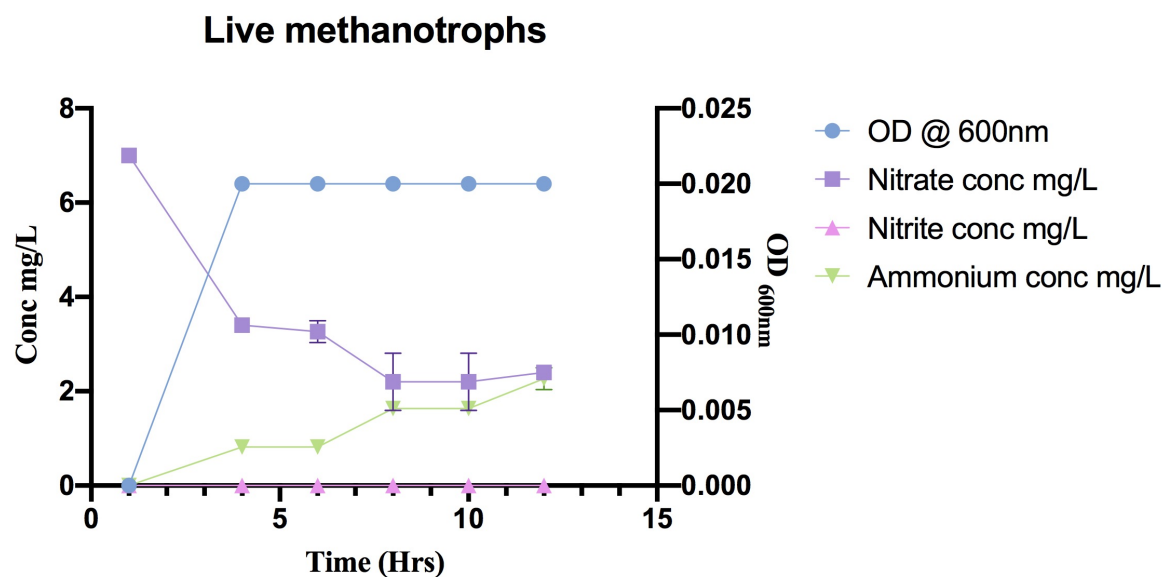


**Fig 3.15:** Change in concentration of nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), ammonia ( $\text{NH}_3^+$ ) and OD over time (A) *P. stutzeri* (B) *P. denitrificans*

### Interpretation

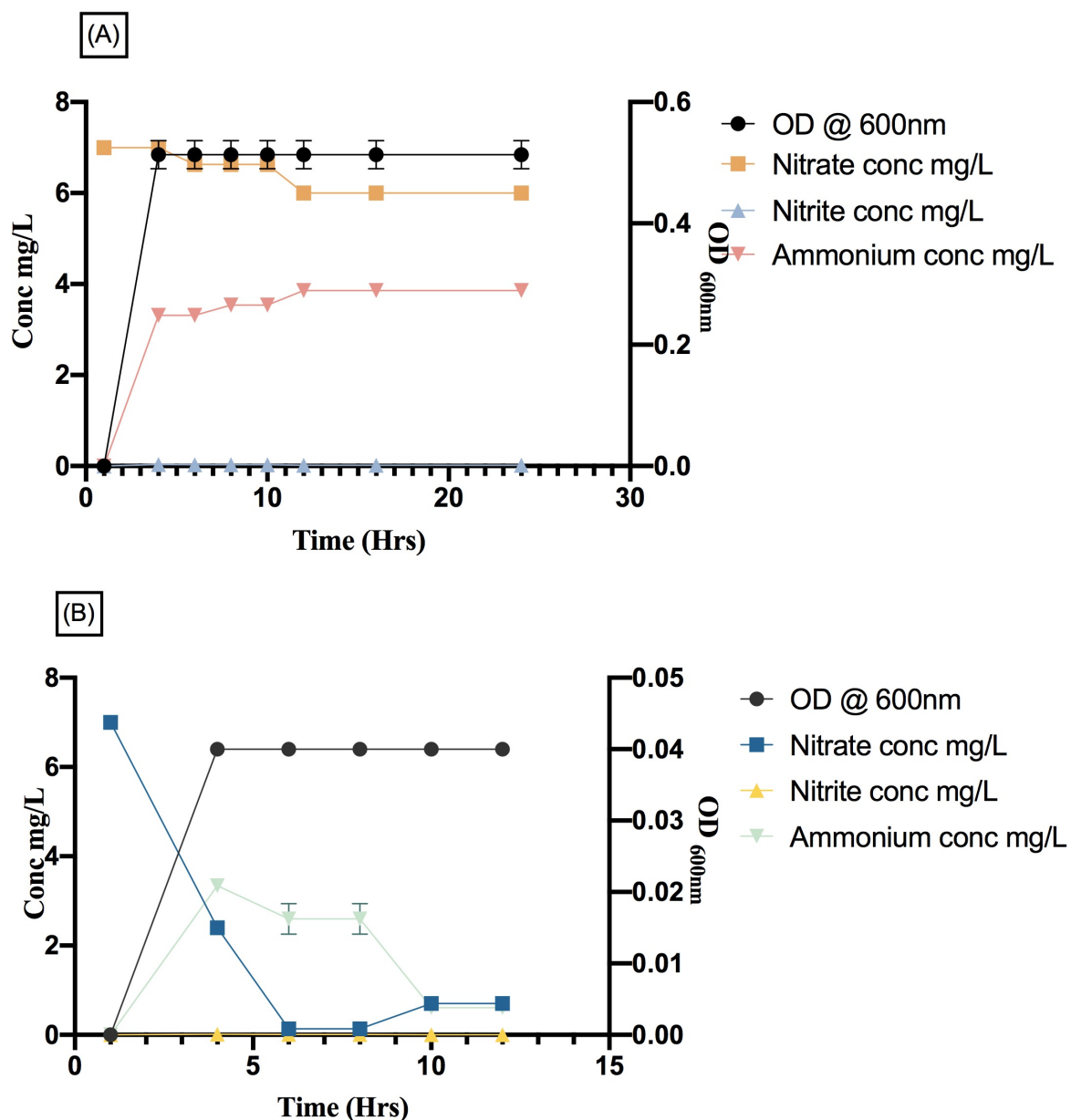
The sudden increase in nitrate concentration towards the end of the exponential phase as observed in the graphs for all the strains when grown in 0.5% (w/v) yeast extract (carbon and energy source) denitrification medium with 7mg/l nitrate. This phenomenon was not observed in denitrification media with sodium citrate (carbon and energy source) and 7mg/l nitrate. This result held true for all the strains with the exception of *P. macerans* that formed aggregates thus interfering with nitrate, nitrite and ammonia concentration analysis.

### 3.6.2 Byproducts of live methanotrophs used as carbon/energy source in denitrification media



**Fig 3.16:** Graph displaying the growth of methanotrophs (control) for the experiments whose graphs are as follows -

3.6.2.1 *P. denitrificans* utilizing methanotrophic biomass and isolated live methanotrophic culture for survival and denitrification

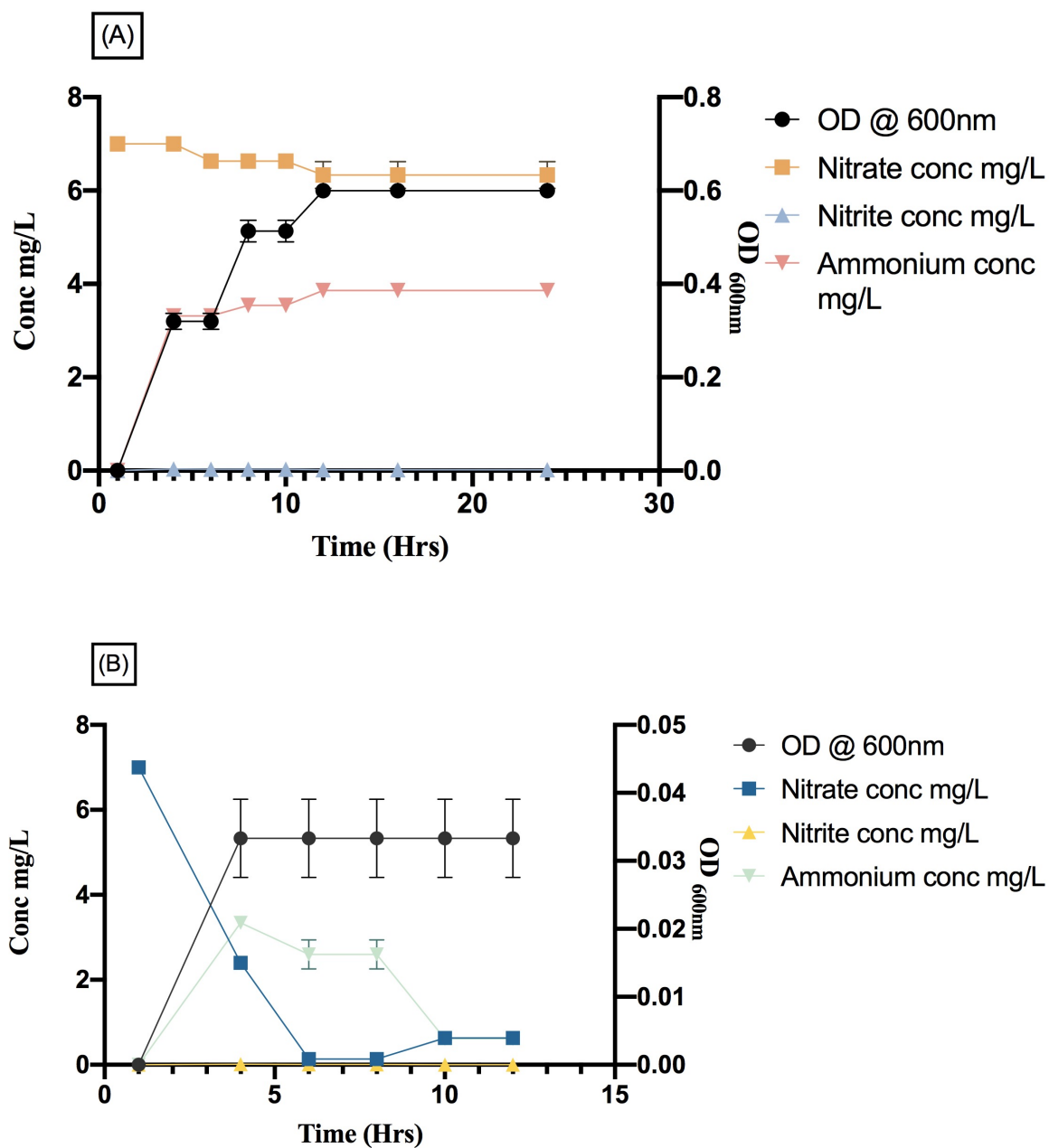


**Fig 3.17:** Change in concentration of nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), ammonia ( $\text{NH}_3^+$ ) and OD over time - *P. denitrificans* using (A) methanotrophic biomass and (B) using live methanotrophic culture<sup>8</sup>.

<sup>8</sup> Incubation temperature- *B. subtilis* 37°C, *P. stutzeri* 37°C, *P. denitrificans* 30°C, *Acidovorax* spp 30°C. and *P. macerans* 30°C.

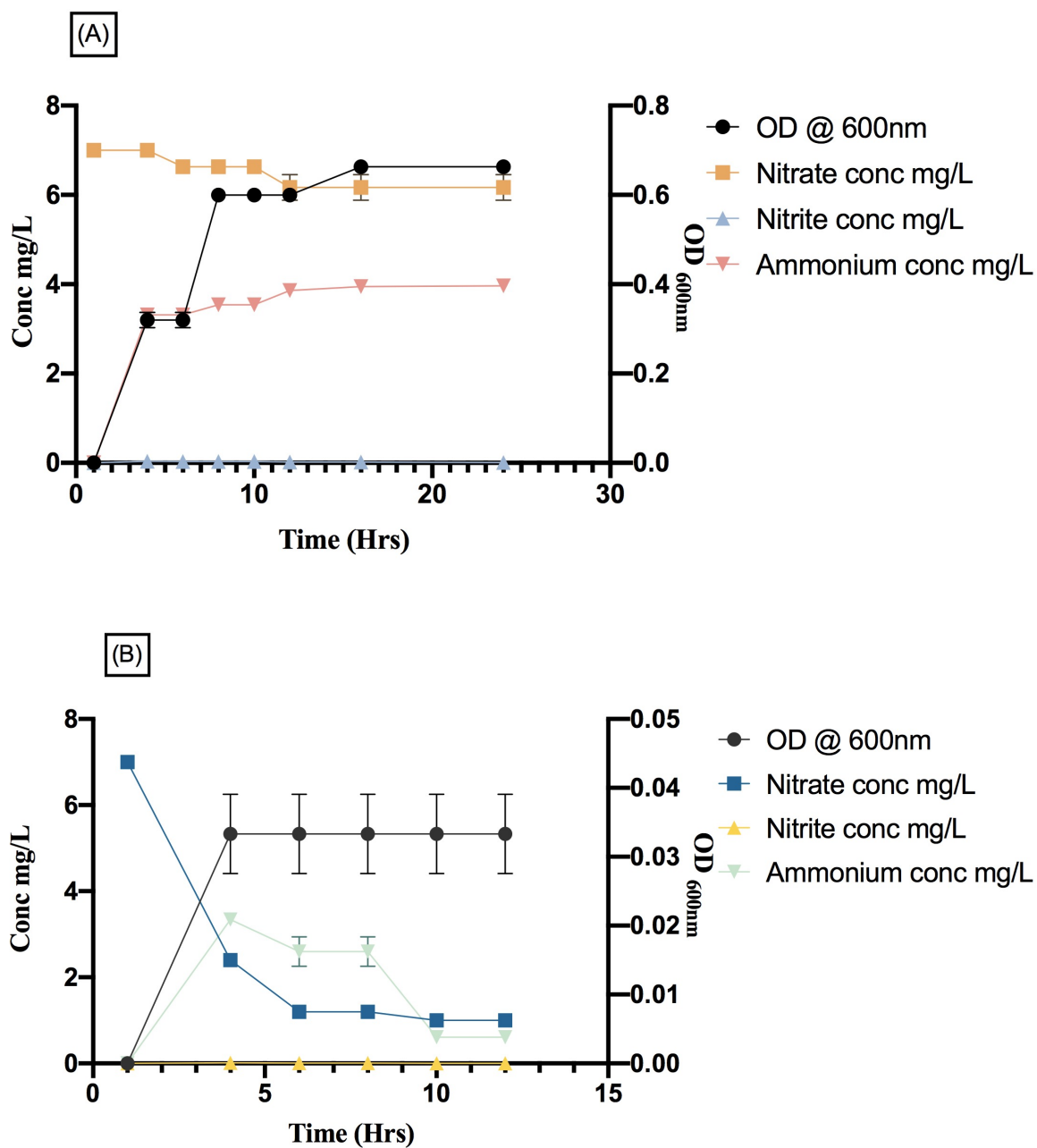
Shaking incubator speed- 200rpm, volume of media- 100ml, volume of inoculant- 1ml

3.6.2.2 *P. macerans* utilizing methanotrophic biomass and isolated live methanotrophic culture for survival and denitrification



**Fig 3.18:** Change in concentration of nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), ammonia ( $\text{NH}_3^+$ ) and OD over time - *P. macerans* (A) using methanotrophic biomass and (B) live methanotrophic culture

3.6.2.3 *Acidovorax spp.* utilizing methanotrophic biomass and isolated live methanotrophic culture for survival and denitrification



**Fig 3.19:** Change in concentration of nitrate, nitrite and ammonia for *Acidovorax spp.* over a period of time. (A) using methanotrophic biomass and (B) live methanotrophic culture



3.6.2.4 *B. subtilis* utilizing methanotrophic biomass and isolated live methanotrophic culture for survival and denitrification

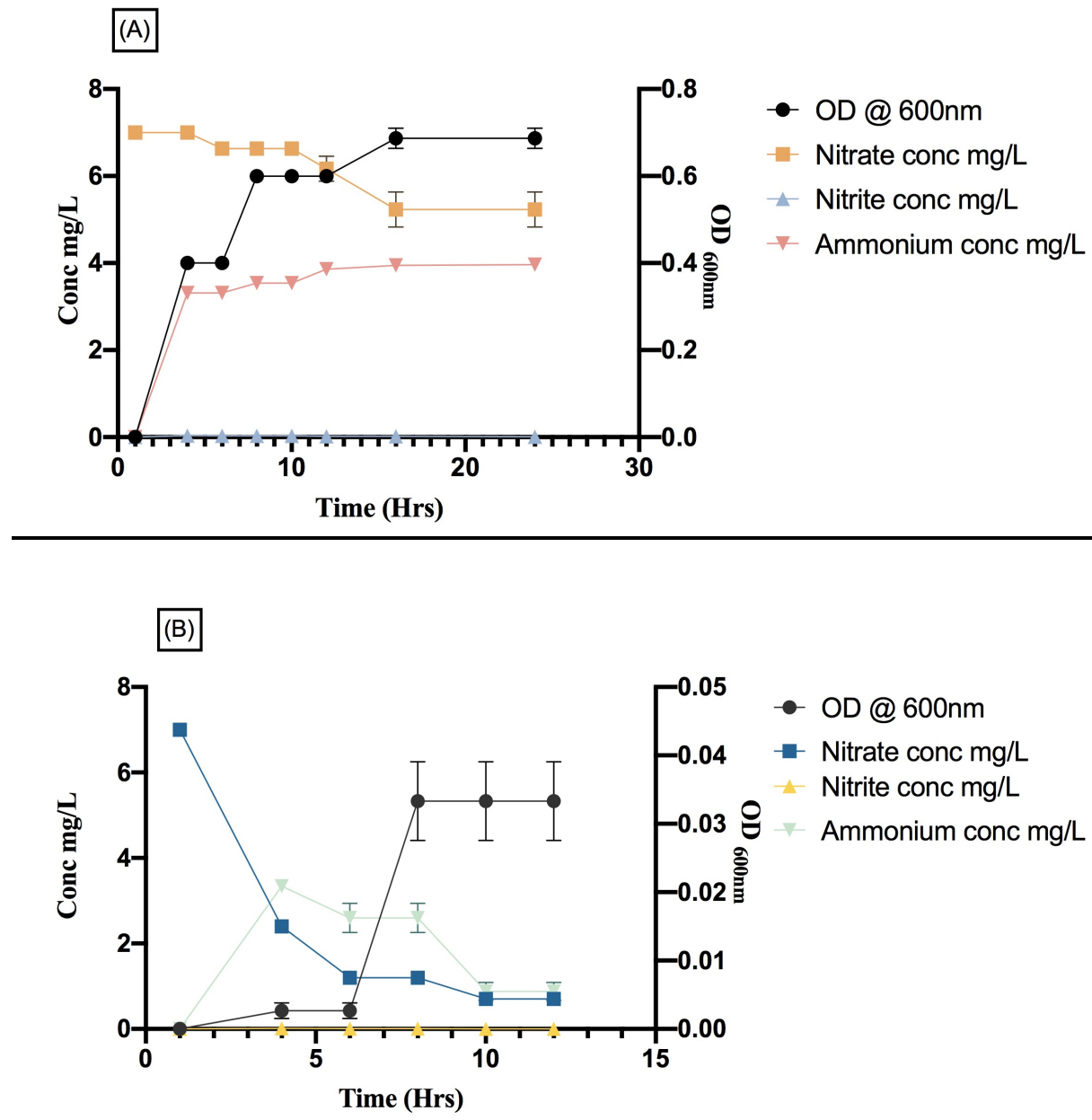
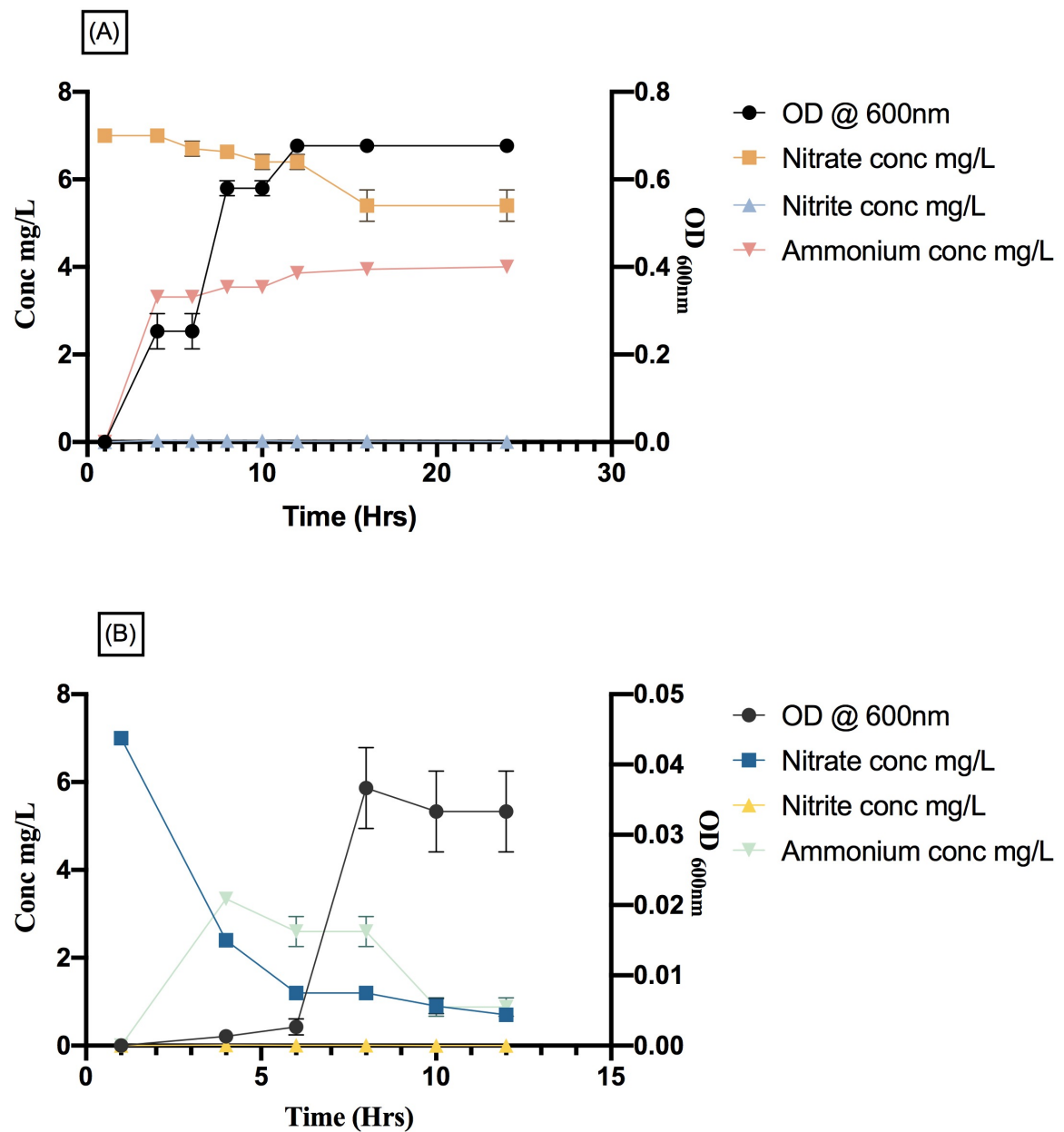


Fig 3.20: Concentration of nitrate, nitrite and ammonia for *B. subtilis* over a period of time. (A) using methanotrophic biomass and (B) live methanotrophic culture

3.6.2.5 *P. stutzeri* utilizing methanotrophic biomass and isolated live methanotrophic culture for survival and denitrification



**Fig 3.21: Concentration of nitrate, nitrite and ammonia for *P. stutzeri* over a period of time (A) using methanotrophic biomass and (B) live methanotrophic culture.**

## **Interpretation**

These graphs address the most important part of this thesis. The first aim was to get these strains of denitrifying bacteria growing with live methanotrophic culture (isolated in the lab) and methanotrophic biomass and the second aim was to test for denitrification. Live methanotrophic culture and live denitrified culture were used as controls for these experiments. All the strains grown with methanotrophic biomass as the sole source of energy and carbon showed very slow reduction in nitrate, little change in concentration of nitrite and rapid increase in concentration of ammonia, suggesting denitrification does occur. In the second set of experiments where the denitrifiers were grown with live methanotrophs, significant reduction in nitrate was observed leading to the possibility of methanotrophs utilizing and reducing nitrate. This assumption was proved correct by the graph for live methanotrophs, which was used by the control.



## Chapter Four

### Discussion

Nitrate is a highly soluble salt leading that can lead to detrimental effects on the ecosystem when observed in higher concentrations. Its environmental presence has been increased in the past century due to anthropogenic sources like the utilization of nitrogen fertilizers, organic waste, and runoff from sewage treatment plants. The unprecedented increase of nitrate burden on the ecosystem has disturbed the aquatic ecosystem, hence calling an urgent need to explore alternatives. Reflecting on ESR/CAPE's current focus on discovering innovative approaches to reduce nitrate in aquifers, in this research, I have tried to explore the potential of denitrifying bacteria to reduce nitrates into molecular nitrogen. These bacteria have a natural ability to reduce nitrates and are typically facultative anaerobes, and only perform denitrification in conditions with absence of oxygen. Recent studies have shown certain denitrifying bacteria that are strictly anaerobic.

The bottlenecks of using live cultures in industrial scale are two-fold – (1) scalability and the (2) cost. I try to answer these questions by (1) studying the growth characteristics of these organisms, and (2) exploring denitrification capacity across different media compositions. We observe that the most optimal denitrification is observed when sodium citrate is used as a carbon source, and sub-optimal denitrification is observed with complex media such as yeast extract or methanotrophic biomass. Simple media such as Lysogeny Broth or Denitrification media (which has sodium citrate) usually tend to be expensive as they are synthetic and have high concentration of carbon source. Complex media, such as yeast extract and methanotrophic biomass can be retrieved as byproducts of industrial processes which can be recycled and hence can serve as a cheaper alternative. An economically viable option can be determined by finding a balance between using a simple media and a complex media. All denitrifying strains studied except *P. macerans*, which formed aggregates were found to be scalable, hence showing strong promise in its future for its use at an industrial scale.

The primary objective of this study was to understand denitrification products formed at a particular phase of the growth cycle during the process of denitrification and make initial inferences about relations between biomass and reduction of nitrate. The second objective was to grow the denitrifiers with methanotrophic biomass, serving as a growth medium and to test for reduction in concentration of nitrate and concentration of other products formed. The third objective was to repeat the same experiment by using a live methanotrophic culture. Growing methanotrophs and denitrifiers together and analyzing the products formed during denitrification, if the process took place.

Several experiments were performed throughout the course of this study to achieve the objectives. The experiments targeting specific objectives are as follows -

Objective one, to understand denitrification products formed by *Bacillus subtilis*, *Paracoccus denitrificans*, *Acidovorax* spp., *P. macerans* and *Pseudomonas stutzeri* during the process of denitrification. Denitrification was tracked by experiments that measured the concentration of nitrate, nitrite and ammonia through growth cycle from lag to constant phase. The second objective, to understand the relationship between biomass and reduction of nitrate was achieved by analyzing the results for cell counts along with denitrification results allows to draw inferences about relation between cells per ml of denitrifying bacteria and reduction in nitrate concentration. The third objective, to culture denitrifying bacteria with methanotrophic biomass, serving as a growth medium and to test for reduction in concentration of nitrate and concentration of other products formed were tested through a series of experiments. The graphs for growth and denitrification of denitrifiers fed with biomass showed decrease in nitrate and increase in ammonia concentration.

The results for all the experiments performed to achieve the aforementioned objectives are discussed in Chapter 3: Results. An overall discussion of the results describing the drawbacks and future experiments to better understand the process of denitrification. This would serve to reduce nitrate contamination from aquifers with least invasion of the aquatic habitat.

## **4.1 Metabolism**

A total of five strains were used in this study to accomplish the three above mentioned objectives and the strains are as follows. *B. subtilis* and *P. stutzeri* both grew at 37°C in all the liquid media used throughout this study. *B. subtilis* grew both aerobically and anaerobically on the contrary *P. stutzeri* which grew only aerobically. *Acidovorax* spp., *P. denitrificans* and *P. macerans* grew at 30°C. All the five strains of denitrifiers showed growth when streaked on nutrient agar during isolation. *Acidovorax* and *P. denitrificans* were cultured successfully on all the liquid media used throughout this study with the exception of *P. macerans*. This particular strain formed aggregates in LB and denitrification media (Chapter 2). Repeated alterations were made in the process of inoculation and incubation. These alterations included agitation of inoculant, increasing the speed of the shaking incubator and using Erlenmeyer flasks with ridges to facilitate better exchange of gases. Despite the alterations the culture still

formed aggregated towards the end of lag phase or during early exponential phase (Fig 3.3 and 3.6). *P. macerans* was able to grow in denitrification media when sodium citrate was replaced with yeast extract as the carbon and energy source (Fig 3.8).

## **4.2 Growth in a nutrient rich media**

To understand the pattern of growth, curves were plotted for all strains cultured in LB at their optimum growth temperature. These were used as a reference to understand good growth for individual strains. This was achieved for all the strains except *P. macerans*. Followed by dilutions of the culture to obtain cells per ml and its OD. The resulting graphs depict a relation between time, OD and number of cells present per ml (carbon source). Understanding this relationship will play a key role in tying the following experiments together. The process of denitrification is dependent on a carbon source and an electron donor. The knowledge associated with the concentration of carbon required to carry out nitrate reduction is crucial to solve the global issue of nitrate burden in aquifers.

## **4.3 The effect of media on denitrification**

Denitrifiers are capable of utilizing a range of carbon compounds like methanol, acetic acid, yeast extract, starch, etc [92]. Research confirms an effect on denitrification (denitrification rates, kinetics, concentration of products formed) with change in carbon source [93]. Justifying the difference in OD (growth of the denitrifying bacteria), concentrations of nitrates, nitrites and ammonia observed for the same strain when cultured in different media where the carbon sources were complex. All the denitrifying strains showed variation in growth rates in the two separate growth media used.

The first set of denitrification experiments were performed using modified denitrification media [94] where yeast extract served as the carbon source to facilitate denitrification. Yeast extract was chosen as the carbon source because it was the closest to methanotrophic biomass in terms of composition complexity. Methanotrophic biomass was used in the following experiments to achieve the aforementioned objective and the results are discussed below.



## **4.4 Sudden increase in nitrate**

Unexpected spikes in the nitrate concentrations were observed towards the late exponential phase for all the strains grown in the modified denitrification media with yeast extract. Chemical oxygen demand came forward as the likely reason during literature review [95]. Studies have reported reduction in chemical oxygen demand (COD) leading to an increase in the concentration of nitrate in the culture. One hypothesis being yeast extract added to the denitrification media could be the reason behind the spike. This hypothesis was confirmed by analyzing the concentration of nitrates, nitrite and ammonia for all the strains growing in denitrification media and using sodium citrate as the carbon source (Fig 3.16 and 3.17). The addition of yeast extract along with other explanations mentioned in this section leading to a cumulative effect of increase in nitrate [96]. Yeast extract is added to various media as a source of nitrogen. The nitrate concentration increases during the late exponential phase, a possible reason could be the utilization of yeast extract by denitrifiers could lead to formation of compounds that might either lead to lysis of the denitrifiers or the compounds could itself be interfering with the reaction process (powder pillows). This hypothesis could also be supported by analyzing the graphs where the increase in nitrate concentration coincides with the beginning of the stationary phase (no growth).

This impacts the ultimate goal of the thesis by indicating that microorganisms, in this study yeast extract and methanotrophs are more than carbon sources and their complex composition needs to be studied to better understand the reactions taking place and the compounds formed that might possibly interfere with the goal of reducing nitrate.

## **4.5 Methanotrophic biomass as a carbon source**

As observed and discussed in detail in the previous section, using yeast extract as the carbon source leads to a sudden increase in nitrate concentration. A similar result was expected when methanotrophic biomass was used as the carbon source. On the contrary all the denitrifying bacteria used in this study showed good growth and reduced nitrate (Fig .3.19 - 3.23). The total decrease in nitrate concentration was nearly 1-1.5mg/L on an average for all the strains used in this study. The reduction must have likely been limited by the concentration of methanotrophic

biomass. Future experiments in this direction could involve increasing and optimizing the concentration of methanotrophs in order to achieve higher denitrification.

## **4.6 Live methanotrophs as a carbon source**

The concept behind using live methanotrophs was as follows -

There are a large number of bacteria present in the aquifers itself. The byproducts from these microorganisms could be consumed by the denitrifying bacteria upon death of microorganisms could which can serve as an additional source of carbon to support the growth of denitrifying bacteria and subsequently, denitrification. This experiment was set up in the following pattern, 1. denitrifiers + methanotrophic biomass, 2. denitrifying bacteria + live methanotrophic bacteria, 3. live methanotrophic bacteria. The results obtained (Fig 3.19 - 3.23) showed significant decrease in nitrate concentration over a very short time span. Although careful analysis of the results indicate nitrate being used by live methanotrophic bacteria and not the solely denitrifiers. The concentration data for live methanotrophs + denitrifiers was similar to concentration data for live methanotrophs suggesting nitrate was utilized by the live methanotrophic culture.

## **4.7 Future prospects**

The experiments that would lead to more clarity with respect to growth of denitrifiers in complex media like yeast extract, methanotrophic bacteria or a mixture of microorganisms would be compositional analysis using different tests [97]. Will help evaluate and monitor the exact nutrients, carbohydrates, proteins, fatty acids involved. This might help manage and predict the concentration of compounds formed as a result of utilization of carbon source and in turn denitrification.

The concentration of nitrate reduced by denitrifying bacteria when methanotrophic biomass was the carbon source being utilized was low. One reason could be the concentration of the methanotrophic biomass becomes limiting. The other experiments along composition analysis would be to increase the composition of biomass being fed to support growth and denitrification.

With respect to culturing live methanotrophs with denitrifying bacteria, the methanotroph that is known to not utilize nitrate, the experiment could be set up in a continuous culture or in a bioreactor due to “experiment time” being an important factor.

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## Appendix A - Abbreviations

### Proteins/Enzymes

1. ATP	Adenosine triphosphate
2. MMO	Methane monooxygenase
3. NADH	Nicotinamide adenine dinucleotide
4. napAB	Nitrate reductase AB
5. narGHI	Nitrate-quinone oxidoreductase
6. nirK	Copper containing nitrate reductase
7. nirS	Nitrate reductase precursor
8. norB	Nitric oxide reductase subunit B
9. norVW	Nitric oxide reductase operon
10. nosZ	Nitric oxide synthase
11. pMMO	Particulate methane monooxygenase
12. sMMO	Soluble methane monooxygenase

### Chemical Pathways

1. RUMP	Ribulose Monophosphate Pathway
2. TCA	Tricarboxylic acid cycle/Kreb's cycle

### Chemical Compounds

1. EDTA	Ethylenediaminetetraacetic acid
2. N <sub>2</sub> O	Nitrous oxide
3. NH <sub>3</sub> /NH <sub>4</sub> <sup>+</sup>	Ammonia/Ammonium
4. NO <sub>2</sub>	Nitrogen dioxide
5. NO <sub>3</sub> <sup>-</sup>	Nitrate
6. O <sub>2</sub>	Oxygen

## Bacteria(s)

- |                            |                                 |
|----------------------------|---------------------------------|
| 1. <i>B. subtilis</i>      | <i>Bacillus subtilis</i>        |
| 2. <i>P. stutzeri</i>      | <i>Pseudomonas stutzeri</i>     |
| 3. <i>P. denitrificans</i> | <i>Paracoccus denitrificans</i> |
| 4. <i>P. macerans</i>      | <i>Paenibacillus macerans</i>   |

## Miscellaneous

- |           |   |
|-----------|---|
| 1. COD    | Chemical Oxygen Demand  |
| 2. CAPE   | Chemical and Process Engineering Department,<br>University of Canterbury – Christchurch, NZ |
| 3. DNA    | Deoxyribonucleic acid   |
| 4. GNS    | Institute of Geological and Nuclear Science,<br>New Zealand                                 |
| 5. HACH   | HACH manufacturing company  |
| 6. LAF    | Laminar Air Flow  |
| 7. LB     | Lysogeny Broth  |
| 8. MCL    | Maximum Contaminant Level   |
| 9. NZ     | New Zealand   |
| 10. OD    | Optical Density   |
| 11. USEPA | U.S. Environmental Protection Agency  |
| 12. UV    | Ultraviolet radiation   |
| 13. WHO   | World Health Organization   |

